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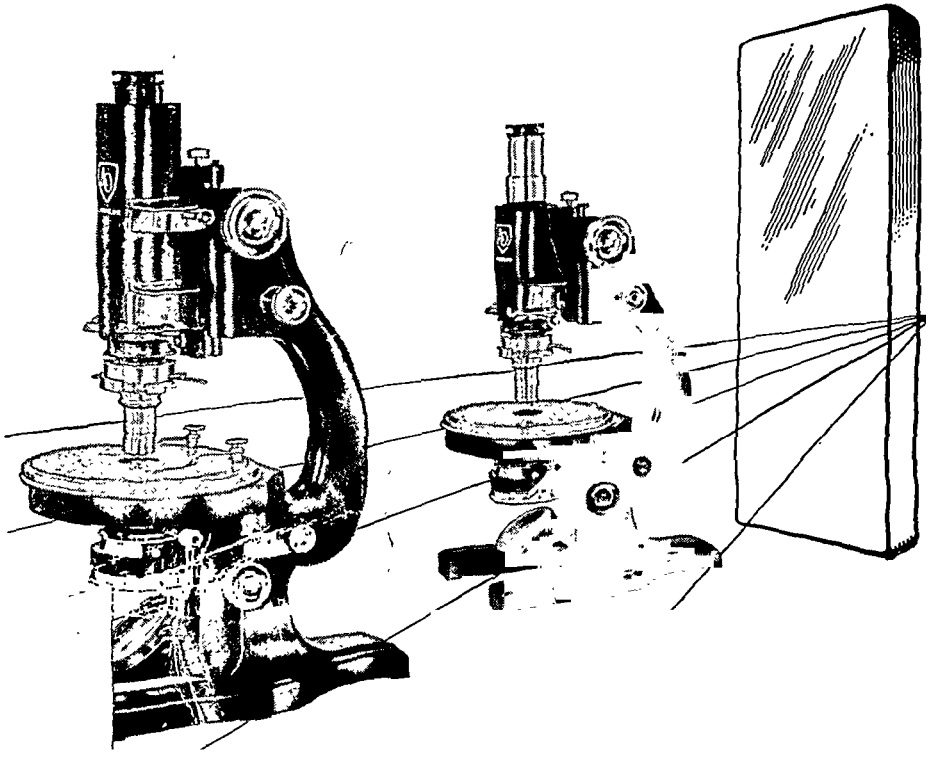

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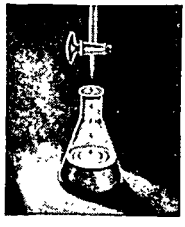

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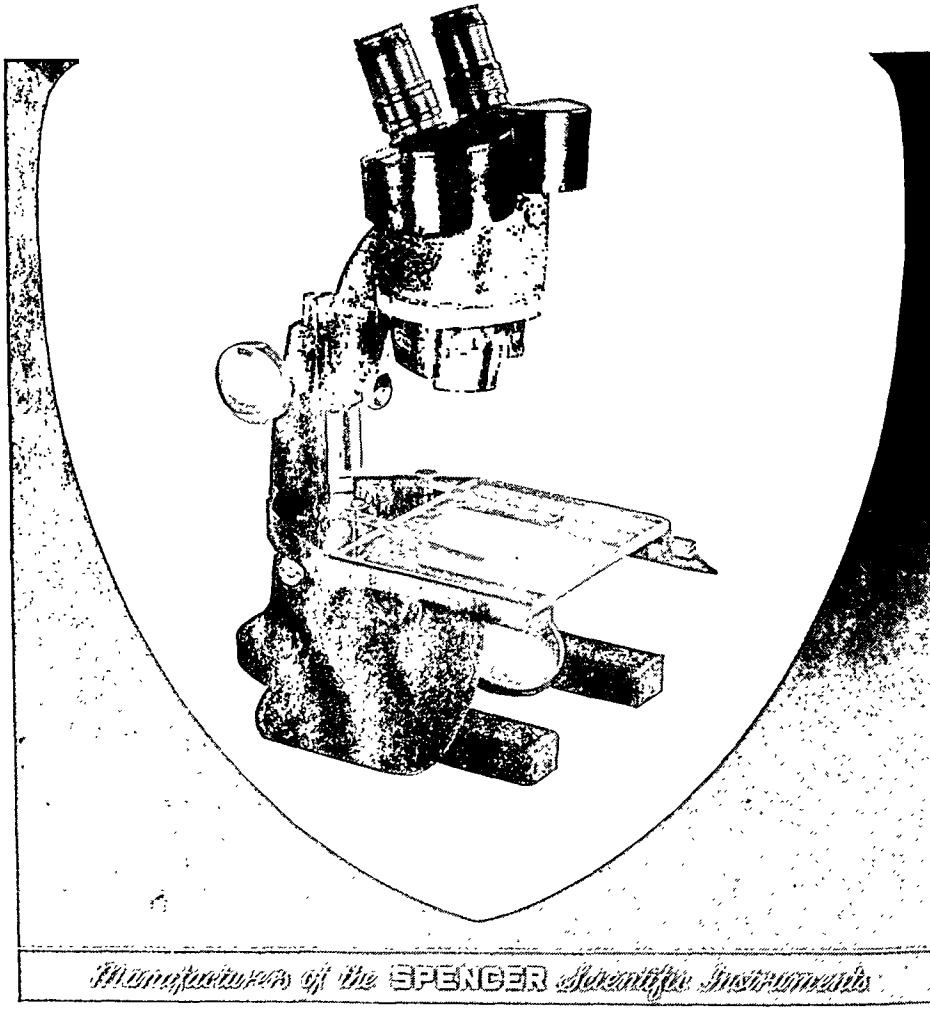

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Pharmacological and Toxicological Studies on Salicylamide*

By C. T. ICHNIOWSKI and W. C. HUEPER

The acute and chronic toxicities, pharmacologic and hematologic effects of salicylamide have been studied. Aspirin served as a comparative control. Salicylamide exerts a moderately quicker and deeper depressing effect than aspirin. Oral daily feeding of one-fourth the single lethal dose for a period of thirteen weeks did not induce any untoward symptomatic and anatomic reactions. Data presented suggest that salicylamide appears to differ in its metabolization from other salicylic compounds. The oral lethal dose of salicylamide in rats equals that of aspirin.

SALICYLAMIDE is a salicylic acid derivative known for approximately fifty years but apparently rarely used as a medicinal agent. While salicylic acid and its derivative, aspirin, are well known for their antipyretic, antirheumatic, and analgesic action, the scanty experimental data available on the pharmacologic effects of salicylamide emphasize its narcotic activity.

Meyer (1) stated that, on tadpoles, salicylamide is more efficient as a narcotic at 3° than at 30°. At both these temperatures salicylamide has a higher "narcotic rating" because of its respective partition coefficients (oil-water) than ethyl alcohol, chloral hydrate, acetone, and benzoic amide. Collett (2) using salicylamide on lower zoological forms (*Loligo, Gonionemus, Perphora*) found that the lipoid theory of narcosis held at weak dilutions, but was complicated by other factors when stronger solutions were employed. Kitano (3) reported progressive narcotic activity with increasing temperatures (up to 39°), when a 1:500 salicylamide solution was tested on the isolated extensor communis muscle of rabbits. Anselmino (4), when investigating the changes in the electromotive activity of collodion membranes by narcotics, observed that variations in potential resulted from the use of salicylamide and compounds of the urethane series.

Because of the possibility that salicylamide might combine the antipyretic and analgesic properties of the ordinary salicylic compounds with narcotic ones, it was decided to study its effects upon warm-blooded

* Received March 27, 1946, from the Warner Institute for Therapeutic Research, New York, N. Y.

animals. Aspirin was used as a comparative control for the experiments.

EXPERIMENTAL

Salicylamide or *o*-hydroxybenzamide is a practically tasteless, yellowish white, crystalline powder that is somewhat more soluble in water than salicylic acid, and which has a melting point at 138°. As it contains an amide group replacing a —OH group in the carboxylic chain, it should be less irritating than either free salicylic acid or sodium salicylate, as the amide group is not likely to split off as readily in the gastric secretion as sodium from sodium salicylate.

Since salicylamide is relatively insoluble in highly aqueous media, several experiments were carried out in order to devise a solvent capable of dissolving salicylamide to a much greater degree than water. Negative results were obtained with a 20% aqueous solution of propylene glycol and a 20% aqueous solution of propylene glycol plus a 20% aqueous solution of ethanol. In a menstruum containing 4 parts of propylene glycol and 1 part of ethanol, as much as a 10% solution of salicylamide could be obtained. However, preliminary experiments with this menstruum indicated that the doses of the menstruum necessary for the administration of adequate amounts of salicylamide would complicate the analysis and interpretation of the narcotic and analgesic action possibly exerted by the drug and in themselves would be irritating because of the hygroscopic nature of these solvents.

Salicylamide solutions prepared by making alkali combinations of the amide in a watery medium using sodium carbonate or triethanolamine left much to be desired, since these solutions turned yellow or yellow-red within an hour and formed a precipitate at times. These changes indicated that decomposition had occurred, making the solutions unsuitable for experimental purposes.

The preparation finally adopted consisted of a suspension of salicylamide in a 0.5% solution of gum tragacanth, which was administered orally and intraperitoneally to rats. Aspirin was given in the same manner.

TABLE I.—TOXICITY OF SALICYLAMIDE AFTER ORAL ADMINISTRATION

Rat Group	Dose, Mg./Kg.	24-Hr. Toxicity	4-Day Toxicity	Comments[a]
1	400	0/5	0/5	Throughout the day of the administration all were responsive and active. On the days following administration the animals behaved normally
2	600	0/5	0/5	Approximately one hour after administration the group was depressed and showed hind leg incoordination. Recovery was noted three hours after administration. On successive days the animals behaved normally
3	800	0/5	0/5	Two of the animals were heavily depressed and gave no response to tail pinches. The other three acted normally. Five hours after administration the whole group was normal and behaved normally on the succeeding days
4	1000	0/5	0/5	Similar to Group 3, but depression lasted until end of work day. Recovery was complete on the following morning. Thereafter the animals were normal in activity and response
5	1600	0/5	0/5	Four of the rats showed heavy depression and the remaining one a mild depression approximately one hour post-administration. Response to tail pinch was negative. Recovery ensued following morning. Thereafter the rats acted normally
6	2000	1/5	1/5	One rat died within two hours post-administration. The remaining 4 were heavily depressed and yielded no response to tail pinch. The effect lasted during work day. Recovery noted by next morning and animals behaved norma ly thereafter

[a] The depression did not appear to be a narcosis-like symptom.

TABLE II.—TOXICITY OF ASPIRIN AFTER ORAL ADMINISTRATION

Group No.	No. of Rats in Group	Dose, Mg./Kg.	24-Hr. Toxicity	4-Day Toxicity	Comments
1	5	400	0/5	0/5	All the surviving animals were in good condition barring a temporary distress in the heavier dosed groups, shortly after administration. Recovery was rapid; within thirty minutes. The animals thereafter and until end of observation period of four days acted normally
2	5	600	0/5	0/5	
3	5	800	1/5	1/5	
4	15	1000	1/15	2/15	
5	10	1600	1/10	1/10	
6	10	2000	0/10	0/10	

A. Acute Toxicity: Oral Administration.—In a preliminary experiment 12 groups of rats, containing 5 animals each, were given doses of 0.4 to 2.0 Gm./Kg. of the two drugs. The salicylamide as

well as the aspirin was administered as a 20% suspension in tragacanth solution by means of a hypodermic syringe with a blunted needle. Six groups were employed for the testing of each drug. The results obtained are tabulated in Tables I and II.

After the approximate lethal dose of salicylamide had been thus ascertained, 3 groups of 15 rats each were tested with dose levels of 0.5, 1.0, and 2.0 Gm./Kg. of salicylamide and aspirin, respectively, given as a 20% suspension in 0.5% tragacanth solution. The results obtained are presented in Tables III and IV.

The autopsies of the rats which were treated with either drug and which died during the period of observation exhibited congestion and hemorrhages of the lungs and hyperemia of the meninges. All other organs were grossly normal.

B. Chronic Toxicity: Oral Administration.—Five groups of 20 rats each (10 males and 10 females) weighing between 100 and 130 Gm. were placed on chronic salicylamide and aspirin medication, respectively. The dose levels of the two drugs given were 5, 25, 50, 100, and 200 mg./Kg., five times per week, for thirteen weeks, with a total of 67 doses.

TABLE III.—LETHAL ORAL DOSE OF SALICYLAMIDE IN RATS

Group No.	Rat No.	Dose, Mg./Kg.	24-Hr. Toxicity	1-Wk. Toxicity	Comments
1	1–15	500	0/15	0/15	Rats showed no evidence of narcosis or depression; they responded normally for several hours after administration and reacted normally for remainder of observation period
2	16–30	1000	0/15	0/15	Rats behaved and reacted like those in Group 1
3	31–45	2000	9/15	9/15	Seven died within one hour after administration; seven went into a heavy depression, while the remaining ones of the group reacted and responded normally. Two more rats died overnight after the administration, giving a 60% mortality in twenty-four hours

TABLE IV.—LETHAL ORAL DOSE OF ASPIRIN IN RATS

Group No.	Rat No.	Dose, Mg./Kg.	24-Hr. Toxicity	1-Wk. Toxicity	Comments
1	40–60	500	0/15	2/15	Animals were in good condition after administration and showed no symptoms of depression. For the period of observation the animals reacted normally
2	61–75	1000	5/15	9/15	No depression for several hours. Deaths overnight
3	76–90	2000	8/15	12/15	No depression for several hours. Deaths overnight

The autopsies performed on the rats of both groups which died during the first week after the administration of the drugs revealed in all animals congestion and edema of the lungs, which findings in some cases were associated with local hemorrhages, and hyperemia of the liver. All other organs were grossly normal. The approximate *LD* for both drugs is thus in the neighborhood of 2 Gm./Kg. Salicylamide appears to cause a distinct depression more quickly than aspirin, which in turn seems to be somewhat more toxic than salicylamide.

Intraperitoneal Administration.—Since the depression noted in the oral salicylamide groups did not appear like a narcosis, it was decided to repeat the administration of salicylamide and aspirin by the intraperitoneal route. A 10% suspension of these drugs in a 0.5% solution of gum tragacanth was injected into four groups of rats, of 10 animals each, at four dose levels ranging from 0.25 to 1.5 Gm./Kg. Tables V and VI show the results of this study.

It is obvious from these data that aspirin intraperitoneally given is more toxic than salicylamide, despite the fact that the latter drug elicits a more rapid and a deeper depression than the former. Throughout the observation period there was no evidence of a narcotic action of salicylamide, even when given in doses of 1000 mg./Kg.

The amounts administered correspond to approximately 1, 5, 10, 20, and 40 times the average human adult dose. The total quantity of salicylamide given to an individual rat ranged between 70 mg. and 13,400 mg., while that of aspirin varied from 75 mg. to 13,800 mg. The volume administered per gram of rat was either 0.01 cc. or 0.02 cc. The medicaments were suspended with the aid of a 0.5 per cent tragacanth solution and given with a syringe.

All animals dying during the course of the experiments or sacrificed afterward were subjected to a post-mortem examination. Some of the surviving rats were sacrificed at the end of the experimental period for hematologic studies, while others were sacrificed by the intravenous injection of a 4% formaldehyde solution three to seven weeks after the cessation of medication. A histologic examination of the internal organs was made of 46 rats which received salicylamide and of 44 rats given aspirin. The hematologic examination consisted of the determination of the prothrombin time, coagulation time, amount of hemoglobin, counts of the leucocytes and erythrocytes, and of differential counts whenever an abnormal number of leucocytes was found. The blood for these tests was obtained from [illegible] jugular vein. The weight of the rats was [illegible] mined weekly for eighteen to twenty weeks.

Deaths occurred during the course of the

ment to about an equal degree in all groups and thus at all dose levels with both drugs. Inasmuch as in all these cases the autopsy showed hyperemic and hemorrhagic lungs and a seropurulent pleurisy and pericarditis, it is likely that these fatalities are not attributable to any toxic action of the chemicals given, but to technical accidents connected with their administration, either represented by a direct injection of the suspension into the lungs or by a secondary regurgitation of the injected matter into these organs. This conclusion is supported by the fact that all rats treated with either drug and killed at the end of the experimental period did not exhibit any significant abnormal changes upon gross and microscopical examination of their internal organs. Neither salicylamide nor aspirin in the doses given thus seemed to have exerted any anatomically demonstrable toxic toxic or lethal effect.

time of the cessation of treatment with salicylamide ranged from 200 to 310 Gm., and fluctuated between 240 and 370 Gm., four weeks later, while the corresponding figures for the female rats were 160 to 205 Gm., and 180 to 235 Gm., respectively. In the male aspirin rats the weights ranged from 250 to 320 Gm., at the cessation of treatment and from 270 to 350 Gm., four weeks later, whereas the corresponding figures for the female rats stood at 180 to 215 Gm., and at 185 to 240 Gm., respectively. It is uncertain whether these differences in the weight curves of the two sexes are merely sex conditioned or represent differences in reaction of the two sexes to a toxic action of the two salicylic compounds.

The hematologic studies showed that the prothrombin times of the 19 rats of the salicylamide series were within normal limits (average from 29.26

TABLE V.—INTRAPERITONEAL TOXICITY OF SALICYLAMIDE

Group No.	Rat No.	Dose, Mg./Kg.	24-Hr. Toxicity	1-Wk. Toxicity	Comments
1	1–10	250	0/10	1/10	All rats were heavily depressed fifteen minutes after administration. Within two hours all 10 rats had recovered. The animals continued in good condition one week after administration
2	11–20	500	2/10	2/10	All were readily depressed in fifteen minutes after administration. Five hours after administration the 8 survivors had recovered and continued in good condition for one week. Two died within two hours after administration. During period of depression the rats exhibited a hind leg paralysis
3	21–30	1000	9/10	9/10	All in the group reacted very readily, going into a heavy depression and coma-like condition. Nine of the rats died; 7 within thirty minutes after administration and the other 2 died within one and one-half hours after administration. The one survivor recovered from the depression within five hours after receiving the drug. This survivor continued in good condition for the remainder of the observation period of one week
4	31–40	1500	10/10	..	All 10 rats went into a heavy depression almost immediately. Seven died ten minutes after administration and the other three within one hour later

TABLE VI.—INTRAPERITONEAL TOXICITY OF ASPIRIN

Group No.	Rat No.	Dose, Mg./Kg.	24-Hr. Toxicity	1-Wk. Toxicity	Comments
1	1–10	250	0/10	0/10	No acute symptoms discernible; all were in good condition and remained thus for one week
2	11–20	500	4/10	9/10	Slight depression set in within two hours after administration; complete recovery three hours later. Survivors after twenty-four hours were in good condition. Four deaths within twenty-four hours; 3 more died during third day; one additional rat died on fourth day, and the ninth rat died on seventh day
3	21–30	1000	10/10	10/10	All ten died within two hours after administration
4	31–40	1500	10/10	10/10	All ten died within three hours after administration

The weights of the rats of both series and of all dose levels increased steadily during the course of treatment to about an equal degree with the exception that the female rats gained weight less rapidly than the male and, particularly, did not show the very rapid elevation of weight after the discontinuation of medication, which was seen in the male rats of both series. The weights of the male rats at the

seconds to 36.55 seconds) after sixteen weeks of medication. The coagulation times were relatively high in many instances with the averages of the different groups fluctuating between five and seven minutes. The erythrocytic and hemoglobin values were at the lower border of the normal range. The number of leucotytes, on the other hand, ranged in three animals between 300 and 5300 and was above

10,000 in only 2 other animals. The differential counts did not reveal any consistent differences between the rats with a normal number of leucocytes and those with a leukopenic reaction.

The prothrombin times of the aspirin rats ranged from averages of 15.8 seconds to those of 33.1 seconds without showing any relation to the dose levels. The coagulation times fluctuated between averages of four minutes and two seconds, and six minutes and thirty-one seconds. The erythrocyte and hemoglobin values were well within the normal range and in some rats were even slightly above the normal limit. The number of leucocytes varied between 7000 and 17,600 and was in most rats above 10,000. The differential counts exhibited normal variations.

In view of the leukopenic reactions observed in a few rats of the salicylamide series, a second chronic experiment was initiated under carefully controlled conditions for the purpose of studying more thoroughly the hematic reactions of rats exposed to salicylamide over long periods of time. Again both salicylamide and aspirin groups were used. The individual groups contained 10 rats (5 males, 5 females). Three dose levels, 5, 50, and 200 mg./Kg., given five times weekly, were used.

The medicaments, as in the previous experiment, were given in 0.5% tragacanth suspension. Blood samples were obtained from the jugular veins of the animals which were kept under light ether anesthesia during the sampling process. A sampling schedule was so arranged that the blood specimens were obtained bi-weekly from both the aspirin and salicylamide rats. The females and males were so alternated that the bi-weekly specimens of blood were obtained and any given rat was not used for sampling but once every four weeks. A 1-cc. sample of blood was obtained from each rat for purposes of the routine hematologic studies which included red and white cell counts, hemoglobin determinations, differential counts, and prothrombin time. A control blood specimen was obtained for analysis from each rat prior to medication. The medication course lasted for thirteen weeks, during which period the rats surviving had received 67 doses. The total amounts received by the survivors of the three groups on each medication of 5 mg., 50 mg., and 200 mg./Kg., per day were 335 mg., 3350 mg., and 13,400 mg., per Kg., respectively. Representative numbers of each group were sacrified after the medication course for gross pathologic examination.

An appreciable number of rats died during the course of the experiment as the result of technical accidents. Several animals in the aspirin series receiving 200 mg./Kg. developed "cauliflower ears" during the latter part of the experiment which were transformed into large blisters of pink to bluish red color and up to a small navy bean size. In several instances these blisters receded after some time and the affected ears became distorted by cicatrization.

The hematological data of both series did not reveal any significant changes from the normal range with the exception that again the rats of the salicylamide group had low normal values as to hemoglobin and erythrocytes, while the animals in the aspirin group revealed higher figures in these respects. There were no leukopenic reactions among the animals treated with salicylamide. The prothrombin times fluctuated considerably during the course of the experiment among the rats of both series, but there was no progressive or consistent trend toward a hypoprothrombinemia.

The post-mortem examination of the rats which died during the course of the experiment showed congested and hemorrhagic lungs and meninges and, often, seropurulent fluid in the thoracic cavities. The histological examination of the organs of the rats sacrificed at the end of the experiment revealed no abnormalities. The "cauliflower ears" exhibited a large central cavity filled with plasma and erythrocytes and lined by endothelial cells. The overlying epidermis was hyperplastic, while the cartilaginous plate which formed a part of the cystic wall in some instances showed signs of liquefaction necrosis. The surrounding tissue contained distended veins and capillaries and hemorrhages. Regressed lesions consisted of an inflammatory granulation tissue with necroses.

C. **Hypoprothrombinemia Study.**—In view of the fact that claims have been made that a chronic and excessive use of aspirin or other salicylates may lead to the development of a hypoprothrombinemia, it became pertinent to determine whether or not salicylamide exhibits this influence and to the same degree as aspirin. Two groups of 25 rats each were therefore placed on a vitamin K deficient diet (casein 18 parts, yeast 8 parts, salts 4 parts, Wesson oil 5 parts, cod-liver oil 2 parts, cerelose 63 parts) for a period of ten days. The rats of one group received then, in about 5 Gm. of a mixture of cooked cornstarch containing 2% cotton seed oil, 100 mg. of aspirin daily. The second group of 25 rats was fed, in the same mixture, 100 mg. of salicylamide. The prothrombin time was determined before the start of these dietary managements and after six, twelve, and eighteen days thereafter. The blood was withdrawn from the jugular vein.

The average prothombin time of the salicylamide rats was 26 seconds before the start of the treatment and stood at 59 seconds at the end of the experimental period. The corresponding figures for the aspirin rats were 25.3 seconds and 53.4 seconds, respectively. There is thus no fundamental difference in the hypoprothrombinemic action of the two salicylic compounds.

D. **Chemical Determination of Salicylamide.**—Some preliminary work was carried out on the quantitative determination of salicylamide in blood for the purpose of being able to follow blood levels and the persistence of the drug in this medium. The colorimetric method of Brodie, Udenfried and Coburn (5) was applied involving the use of ethylene dichloride for the extraction of the "salicyl" preparation from the blood.

While the method worked well with sodium sal-

icylate and aspirin, doses up to 300 mg./Kg. of salicylamide given orally to dogs failed to give a positive reaction in the blood. The same negative result was obtained when other organic solvents (chloroform, benzol, carbon tetrachloride), possessing a different partition coefficient, were tried. Despite the fact that salicylamide contains a phenolic hydroxyl complex in its molecule like the other two salicylates, it differs in this respect from the ordinary salicylic acid compounds.

DISCUSSION

The various experiments showed that salicylamide has a stronger depressive action than aspirin, but does not elicit a state of narcosis as claimed on the basis of results obtained in *in vitro* experiments and in studies on isolated tissues. The oral lethal dose of salicylamide and aspirin for rats is in the neighborhood of 2000 mg./Kg., while the intraperitoneal lethal dose lies around 1000 mg./Kg. The lethal effect is associated with circulatory disturbances causing a congestion and hemorrhages of the lungs and meninges.

The prolonged, repeated oral administration of large doses of salicylamide and aspirin did not cause in rats any ulcerative lesions of the gastric mucosa, such as those reported by Barbour and Dickerson (6). Such reactions were seen regularly, on the other hand, in rabbits and dogs receiving daily by mouth 0.7 Gm./Kg. of aspirin for four to eighteen days, confirming thereby observations of Thompson and Dragstedt (7), and Barbour and Porter (8), made in dogs. Madisson (9) attributed this effect of salicylates to a phenol-like action of these chemicals upon mucous membranes.

The hematic studies indicate that salicylamide exerts approximately the same hypoprothrombinemic effect as aspirin when given to rats kept on a low vitamin K diet. No such action was observed when these animals received stock diet. The presently available data do not permit a definite decision of the question as to whether or not salicylamide sometimes may produce a leukopenic reaction. The probability of the actual occurrence of such reactions, however, appears to be remote, if salicylamide reacts in this respect like other salicylic compounds which rarely or never exert such a response [Hawkinson and Kerr (10)].

The absence of any other significant untoward effect upon the blood and internal organs when salicylamide is given orally to rats in large doses over long periods of time placed this chemical alongside aspirin, which was shown to be equally harmless under similar experimental conditions [Robinson, Ellis, and Warner (11)]. The outcome of the chemical tests performed on the blood of dogs which had ingested appreciable amounts of salicylamide indicate, on the other hand, that this drug differs fundamentally in its *in vivo* reactivity from that of other salicylates.

CONCLUSIONS

1. Salicylamide exerts a moderately quicker and deeper depressing effect than aspirin.
2. The oral lethal dose of salicylamide in rats equals that of aspirin (2000 mg./Kg.).
3. Rats given one-fourth of the single lethal dose (SLD_{50}) daily for thirteen weeks do not exhibit any untoward symptomatic and anatomic reactions.
4. Salicylamide seems to differ in its metabolization from other salicylic compounds.

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The Anesthetic Effects and Toxicities of Some New Barbituric Acid Derivatives Administered to Mice and Rats. I*

By WILLIAM H. HUNT,† RUSSEL J. FOSBINDER, and O. W. BARLOW

Extensive data are reported on the time of onset of hypnosis, duration of action, and the toxicity to mice and rats for approximately 150 new 5,5-disubstituted barbituric and thiobarbituric acids. In general, the data indicate that the duration of hypnotic effect of the new compounds decreased as the molecular weight increased, and the barbiturates containing a sulfur-interrupted carbon chain in one of the substituent groups were more active than those not containing sulfur.

A SERIES of approximately 150 new 5,5-disubstituted barbituric and thiobarbituric acids, containing a sulfur interrupted carbon chain in one of the substituent groups, were prepared in the laboratories of one of us for the purpose of pharmacological examination. Walter, Goodson, and Fosbinder (1), in a series of papers published elsewhere, have described the methods of preparation of the new compounds and the appropriate intermediates.

All of the compounds prepared by Walter, *et al.*, have been examined experimentally by administration of the sodium salts to one or more species of animal. The data presented in this paper are confined to observations on the rat and the mouse and include information relative to the onset of hypnosis, the duration of effect as determined by various criteria, and the acute toxicity following intravenous injection in the rat and intraperitoneal injection in the mouse. Several of the new derivatives have been administered orally to rats in lethal and sublethal doses and the tolerance of this species of animal to repeated doses was determined.

EXPERIMENTAL

Duration of Effect and Toxicity—Rat.—Solutions of the sodium salts of the barbituric and thiobarbituric acids listed in Tables I, II, III, and IV, respectively, were injected in graded doses into the saphenous vein of male, albino adult rats weighing between 100 and 225 Gm. The volume of the dose administered was kept approximately constant by adjusting the concentration between 1 and 5%. In every instance a total of 0.1 cc. of solution was injected in an interval of fifteen seconds. Adult male rats were used throughout the experiments as other investigators (2, 3) have shown that inconsistent results are obtained with immature animals and that female rats exhibit a more prolonged hypnosis as compared to males.

The onset of hypnosis and the duration of various stages of anesthesia were determined by observing the abolition and reappearance of reflexes as described below:

Righting Reflex—the period during which the animal failed to right itself when placed on its side.

Corneal Reflex—response to the application of the blunt end of a feather applied to the cornea.

Ear Reflex—response to the application of the tip of a feather to the inner surface of the ear.

Tail Reflex—response to immersion of the tail in water at 58–60° for five seconds.

Although the period of loss of righting reflex ended when the animals were able to right themselves, the total period of barbiturate effect was often much longer as indicated by continued depression. In the determination of the mean hypnotic dose (MHD_{50}) the abolition of the ear reflex rather than of the righting reflex was employed as a criterion because a more consistent response was observed. All animals given sublethal doses of the compounds were used only after a seven-day rest period to permit full recovery from the effects of medication. In this study Pentobarbital Sodium, Evipal Sodium, and Pentothal Sodium were used as reference compounds.

It will be seen from Tables I, II, III, and IV that when injected intravenously the new compounds induced characteristic hypnotic effects in common with the reference barbiturates. As is to be expected in an extensive series of derivatives, wherein freedom of substitution is substantial, wide variations in duration of effect, activity, and toxicity were obtained. Inasmuch as data on only one species of animal are being reported here, generalizations concerning the relationship of structure to pharmacologic action will be reserved for a later publica

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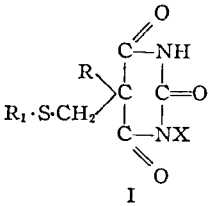
Table V shows the results obtained with a selected group of compounds administered orally to rats in hypnotic and lethal doses. Male adult rats weighing between 175 and 300 Gm. were fasted for eighteen hours and graded doses of the sodium salts were then given by stomach tube. In the lower dosage range 5% solutions were employed, while the concentration was increased to 10% in the lethal range in order to restrict the volume. Five or 10 animals were used at each dosage level and the LD_{50} was estimated by the application of Kärber's method (4).

The formulas of the new compounds referred to by numbers in Tables I, II, III, IV and V are the following: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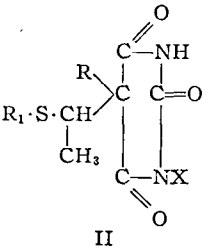
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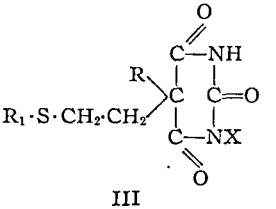
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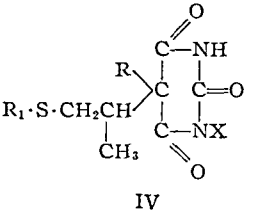
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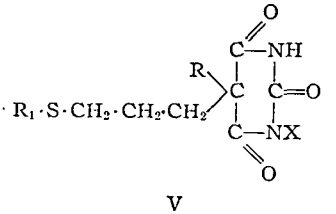
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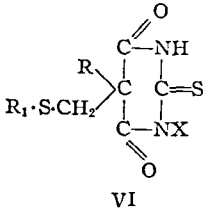
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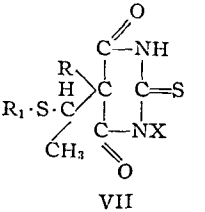
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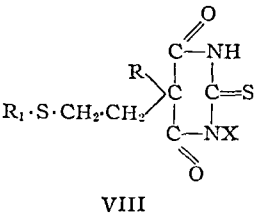
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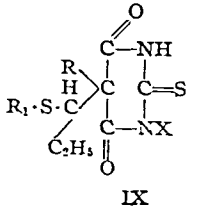
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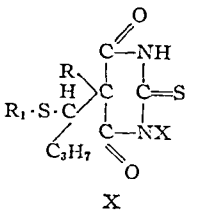
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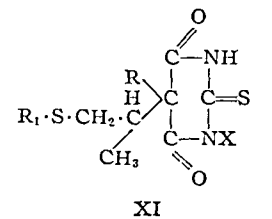

XI

TABLE I.—EFFECTS OF INTRAVENOUS INJECTIONS IN WHITE RATS OF VARIOUS SUBSTITUTED BARBITURIC ACID DERIVATIVES AS HYPNOTICS AND THE OBSERVED TOXICITY

Compound I		No. of Animals	Dose, Mg./Kg.	Down After Injection, Min.	Duration of Loss of Various Reflexes, Min.				Killed
R	R_1				Righting	Corneal	Ear	Tail	
Butyl	Ethyl	5	80	Immediate	67	+	8	+	0/5[a]
Allyl	Ethyl	6	40	Immediate	24	+	+	+	0/6
		6	60	0.7	72	+	22	+	0/6[b]
		6	80	1	93	32	28	18	0/6
		6	100	Immediate	125	39	104	31	0/6[b]
		6	120	0.9	133	20	62	37	2/6
Isoamyl	Ethyl	9	40	Immediate	8	+	+	+	0/9
		12	60	Immediate	12	+	+	+	0/12[c]
		12	80	Immediate	25	+	+	+	0/12[c]
		6	100	Immediate	30	+	2	+	0/6[c]
		12	120	Immediate	42	2	8	2	5/12
		6	140	Immediate	75	15	16	10	5/6
Isopropyl	Ethyl	6	60	Immediate	98	10	37	+	0/6
		6	80	Immediate	109	21	59	+	0/6
		6	100	Immediate	165	79	109	46	2/6
		6	120	Immediate	170	67	116	70	1/6
		6	140	Immediate	188	117	133	111	3/6
Isobutyl	Ethyl	6	20	Immediate	7	+	+	+	0/6
		6	40	Immediate	25	+	+	+	0/6
		6	60	Immediate	55	3	9	3	0/6
		6	80	Immediate	52	10	23	10	0/6
		6	100	Immediate	146	82	93	69	1/6
		6	120	Immediate	95	32	41	30	0/6
		6	140	Immediate	102	87	124	84	3/6
Secondary Butyl	Ethyl	6	20	Immediate	12	+	+	+	0/6
		6	30	0.5	37	+	+	+	0/6
		12	40	Immediate	30	+	+	+	0/6
		12	60	Immediate	50	+	9	+	0/6
		12	80	Immediate	82	26	41	37	1/6
		12	100	Immediate	140	68	75	83	1/6
		6	120	Immediate	263	133	175	173	2/6
		6	140	Immediate	276	105	175	194	1/6
		6	160	Immediate	272	175	175	159	2/6
1-Methylbutyl	Ethyl	6	20	1	7	+	+	+	0/6
		6	40	Immediate	25	+	3	+	0/6
		7	60	Immediate	52	9	29	+	0/7
		6	80	Immediate	67	29	40	13	0/6
		6	100	Immediate	99	51	70	36	0/6
		6	120	Immediate	121	57	74	67	3/6
		6	140	Immediate	139	71	85	69	5/6
Isobutyl	Methyl	6	60	Immediate	44	+	+	+	0/6
		6	80	Immediate	80	26	40	+	0/6
		12	100	Immediate	121	62	87	26	0/12
		12	120	Immediate	141	66	81	48	0/12
		12	140	Immediate	229	138	152	128	2/12
		18	160	Immediate	144	89	105	75	2/18
		6	170	Immediate	95	45	65	41	1/6
		12	180	Immediate	149	89	96	75	8/12
Secondary Butyl	Butyl	6	20	Immediate	13	1	4	+	0/6
		6	30	Immediate	16	+	7	+	0/6
		6	40	Immediate	23	5	14	4	0/6
		6	50	Immediate	29	7	19	11	0/6
		6	60	Immediate	35	14	17	14	0/6
		6	70	Immediate	51	12	24	16	0/6
		6	80	Immediate	...	...	...	...	6/6
Butyl	Butyl	6	20	Immediate	2	+	+	+	0/6
		12	40	Immediate	7	+	1	+	0/2
		6	60	Immediate	10	+	5	+	0/6
		6	80	Immediate	16	3	8	+	2/6
Ethyl	Amyl	6	40	Immediate	12	+	+	+	0/6
		6	60	Immediate	29	9	15	+	0/6
		6	80	Immediate	63	25	43	3	0/6
		6	100	Immediate	88	41	66	17	1/6
		6	120	Immediate	110	71	80	30	2/6
		6	140	Immediate	104	75	75	44	5/6
Isopropyl	Butyl	6	10	Immediate	4	+	+	+	0/6

[a] Constant muscle tremors [b] Slight tremor [c] Convulsive movement on injection

(Cont'd on next page)

TABLE I.—EFFECTS OF INTRAVENOUS INJECTIONS IN WHITE RATS, ETC., (*Cont'd from page 233*)

Compound I R	Compound I R_1	No. of Animals	Dose Mg./Kg.	Down after Injection, Min.	Duration of Loss of Various Reflexes, Min. Righting	Corneal	Ear	Tail	Killed
Isopropyl	Butyl	6	20	Immediate	18	+	6	+	0/6
		6	40	Immediate	25	3	11	+	0/6
		6	60	Immediate	32	20	25	17	0/6
		6	80	Immediate	51	29	44	27	1/6
		6	100	Immediate	64	29	40	29	4/6
Allyl	Butyl	6	10	Not Down	+	+	+	+	0/6
		6	20	Immediate	4	+	+	+	0/6
		6	40	Immediate	9	+	7	+	0/6
		6	60	Immediate	15	5	10	+	0/6
		6	80	Immediate	36	24	25	12	0/6
		6	100	Immediate	74	45	45	23	2/6
Allyl	1-Methylbutyl	6	20	Immediate	3	+	2	+	0/6
		6	40	Immediate	21	2	15	1	0/6
		6	60	Immediate	76	20	42	18	0/6
		6	80	Immediate	51	19	32	19	2/6
Ethyl	Allyl	6	40	Not Down	+	+	+	+	0/6
		6	60	2	76	+	10	+	0/6
		6	80	Immediate	80	14	92	9	1/6[d]
		6	100	Immediate	155	68	126	89	3/6
Ethyl	Isoamyl	6	20	Immediate	15	+	4	+	0/6
		6	30	Immediate	15	+	5	+	0/6[e]
		6	40	Immediate	26	8	13	6	0/6
		6	50	Immediate	25	7	18	4	0/6
		6	60	Immediate	47	12	16	9	2/5
Ethyl	Hexyl	6	20	Immediate	9	+	1	+	0/6
		6	30	Immediate	18	+	11	+	0/6
		6	40	Immediate	60	25	37	21	1/6
		6	50	Immediate	43	34	35	10	1/6
		6	60	Immediate	111	35	59	35	2/6
Δ-1-Cyclohexenyl	Ethyl	6	80	Immediate	91	+	+	+	0/6[f]
Ethyl	2-Ethylbutyl	6	40	Immediate	10	+	2	+	0/6
		6	50	Immediate	13	+	3	+	0/6
		6	60	Immediate	19	2	14	+	0/6
		6	80	Immediate	30	4	18	+	0/6
		6	100	Immediate	38	19	28	+	0/6
		6	120	Immediate	65	26	44	6	1/6
		3	140	Immediate	...	...	...	...	3/3
		3	160	Immediate	...	...	...	...	3/3
Ethylthioethylene	Ethyl	6	80	Immediate	23	+	+	+	0/6
		6	100	Immediate	36	5	9	+	0/6
		6	140	Immediate	63	19	24	6	2/6
		6	160	Immediate	63	22	25	15	3/6
1-Methylbutyl	Allyl	6	20	Immediate	10	+	+	+	0/6
		6	40	Immediate	47	2	10	+	0/6
		6	60	Immediate	70	20	27	8	0/6
		12	80	Immediate	61	23	28	12	0/6
		6	90	Immediate	52	13	23	9	0/6
		6	100	Immediate	108	50	62	45	2/6
		6	110	Immediate	60	30	35	22	4/6
Secondary Butyl	Methyl	6	100	Immediate	147	65	95	25	2/6
		6	120	Immediate	190	106	131	102	1/6
		6	140	Immediate	256	162	197	152	1/6
		6	160	Immediate	317	273	289	253	2/6
1-Methylbutyl	Methyl	6	40	Immediate	30	+	7	+	0/6
		6	50	Immediate	45	14	30	+	0/6
		6	60	Immediate	66	37	50	18	0/6
		12	70	Immediate	54	29	40	13	1/12
		12	80	Immediate	73	31	39	20	2/12
		12	90	Immediate	89	53	63	44	4/12
		12	100	Immediate	65	39	51	28	6/12
β-Methylallyl	Methyl	1	20	Not down	+	+	+	+	0/1
		1	30	Not down	+	+	+	+	0/1[g]
		8	40	3	31	+	+	+	0/8[h]
		10	60	Immediate	62	+	36	+	0/10[i]

[d] Tremors on awakening
[e] Twitched ears after injection
[e] Respiration irregular after injection
[h] Convulsive jerks during injection
[f] Labored respiration on injection; convulsions on awakening
[i] Chewing movements, shakes head

TABLE II.—EFFECTS OF INTRAVENOUS INJECTIONS IN WHITE RATS OF VARIOUS SUBSTITUTED BARBITURIC ACID DERIVATIVES AS HYPNOTICS AND THE OBSERVED TOXICITY

Compound II R	Compound II R_1	No. of Animals	Dose, Mg./Kg.	Down After Injection, Min.	Duration of Loss of Various Reflexes, Min. Righting	Corneal	Ear	Tail	Killed
Ethyl	Butyl	6	10	Immediate	3	+	+	+	0/6
		6	20	Immediate	29	2	8	+	0/6
		6	40	Immediate	25	5	10	+	0/6
		6	60	Immediate	43	13	22	2	1/6
		6	80	Immediate	69	35	37	32	1/6
		6	100	Immediate	...	...	...	...	6/6
Allyl	Butyl	6	20	Immediate	17	+	6	+	0/6
		6	40	Immediate	23	10	13	10	0/6
		6	60	Immediate	37	18	19	18	0/6
		6	80	Immediate	32	10	19	14	2/6
Ethyl	Amyl	6	20	Immediate	14	1	4	+	0/6
		6	30	Immediate	25	7	10	+	0/6
		6	40	Immediate	21	10	11	+	0/6
		6	50	Immediate	41	12	22	5	0/6
		6	60	Immediate	38	33	31	14	0/6
		6	80	Immediate	94	45	58	24	2/6
		6	100	Immediate	...	...	...	...	6/6
Ethyl	Isoamyl	6	20	Immediate	21	+	8	+	0/6
		6	30	Immediate	19	+	8	+	0/6
		6	40	Immediate	31	2	16	3	0/6
		6	50	Immediate	33	5	16	5	0/6
		6	60	Immediate	45	24	26	23	2/6
Isobutyl	Allyl	10	15	Immediate	21	+	1	+	0/10[a]
		10	20	Immediate	13	+	1	+	0/10[b]
		10	25	Immediate	28	+	7	+	0/10
		6	40	Immediate	28	+	7	+	0/6
		6	60	Immediate	71	16	25	19	0/6
		6	70	Immediate	50	17	26	1	0/6
		16	80	Immediate	123	31	41	23	1/16
		6	90	Immediate	62	30	36	23	1/6
		16	100	Immediate	191	74	98	62	5/16
		6	110	Immediate	65	18	24	16	5/6
		10	115	Immediate	283	94	142	58	6/10
Propyl	Isopropyl	6	80	Immediate	45	3	8	3	0/6[e]
Propyl	Methyl	6	80	Immediate	62	+	+	+	0/6
		6	100	Immediate	81	34	40	2	0/6
		6	120	Immediate	101	39	70	3	0/6[d]
		6	140	Immediate	149	94	101	69	1/6[d]
		6	160	Immediate	184	147	161	139	1/6[d]
		6	180	Immediate	178	116	167	146	0/6[d]
		6	200	Immediate	267	176	189	138	2/6[d]
		6	220	Immediate	259	184	191	181	4/6[d]
Ethyl	Ethyl	6	60	Immediate	75	+	+	+	0/6
		6	80	Immediate	141	30	51	3	0/6
		6	100	Immediate	230	102	141	94	1/12
		6	120	Immediate	218	89	116	10	1/6
		6	140	Immediate	204	140	193	130	2/6
		6	160	Immediate	324	245	277	219	3/6
Allyl	Methyl	6	80	Immediate	126	+	43	+	0/6[e]
Amyl	Methyl	6	60	Immediate	38	+	6	+	0/6
		6	80	Immediate	38	10	22	+	0/6
		6	100	Immediate	68	26	40	11	0/6
		6	120	Immediate	76	34	39	13	3/6
Isopropyl	Butyl	6	20	Immediate	13	+	3	+	0/6[f]
		6	30	Immediate	19	+	11	+	0/6[f]
		6	40	Immediate	28	4	17	+	0/6
		6	50	Immediate	44	21	28	16	1/6[g]
		6	60	Immediate	56	28	36	21	1/6[g]
		6	80	Immediate	...	...	...	...	6/6
Amyl	Ethyl	5	60	Immediate	18	+	4	+	0/5[h]

[a] Convulsive movements—2 animals
[b] Movement, lower jaw—2 animals
[c] Convulsions
[d] Twitching and scratching on awakening
[e] Convulsions immediately after injection
[f] Convulsive twitch
[g] Convulsive twitch and scratching
[h] Convulsive jerk at injection

(Cont'd on next page)

TABLE II.—EFFECTS OF INTRAVENOUS INJECTIONS IN WHITE RATS OF VARIOUS SUBSTITUTED BARBITURIC ACID DERIVATIVES AS HYPNOTICS AND THE OBSERVED TOXICITY. (*Cont'd from page 235*)

Compound II R	Compound II R1	No. of Animals	Dose Mg./Kg.	Down After Injection, Min.	Duration of Loss of Various Reflexes, Min. Righting	Corneal	Ear	Tail	Killed
Amyl	Ethyl	6	70	Immediate	26	2	7	+	2/6[i]
		6	80	Immediate	29	2	11	+	2/6[i]
		6	90	Immediate	29	10	16	3	1/6[i]
		6	100	Immediate	38	15	18	9	3/6[i]
Allyl	Isobutyl	6	30	Immediate	7	+	1	+	0/6
		6	40	Immediate	15	+	7	+	0/6
		6	50	Immediate	15	+	7	+	0/6
		6	60	Immediate	22	8	12	4	1/6
		6	70	Immediate	43	22	29	19	4/6
		6	80	Immediate	41	17	22	12	5/6
Isobutyl	Ethyl	6	40	Immediate	23	+	3	+	0/6
		6	50	Immediate	26	+	10	+	0/6
		6	60	Immediate	37	+	15	+	0/6
		6	70	Immediate	29	4	15	+	0/6[j]
		6	80	Immediate	55	26	33	13	0/6[k]
		6	90	Immediate	54	24	28	13	2/6[k]
		6	100	Immediate	74	41	54	33	5/6[k]
Butyl	Ethyl	6	60	Immediate	31	+	2	+	0/6
		6	70	Immediate	44	1	7	+	0/6
		6	80	Immediate	73	17	23	3	0/6
		6	90	Immediate	86	18	23	9	2/6
		6	100	Immediate	72	23	26	19	2/6
		6	110	Immediate	85	25	35	18	4/6
Ally.	Ethyl	6	40	Immediate	32	+	1	+	0/6
		6	50	Immediate	60	+	9	+	0/6
		6	60	Immediate	84	17	40	10	0/6
		6	70	Immediate	131	43	63	14	0/6
		6	80	Immediate	150	70	90	25	0/6
		6	90	Immediate	179	90	118	68	0/6
		6	100	Immediate	183	111	127	97	0/6
		6	110	Immediate	121	69	74	64	0/6
		6	120	Immediate	174	96	114	82	3/6
Allyl	Propyl	6	30	Immediate	15	+	5	+	0/6
		6	40	Immediate	23	2	11	+	0/6
		6	50	Immediate	27	3	13	+	0/6
		6	60	Immediate	31	14	20	2	1/6[l]
		6	70	Immediate	35	18	24	10	1/6[l]
		6	80	Immediate	38	20	25	16	1/6[l]
		6	90	Immediate	46	23	28	18	1/6[l]
		6	100	Immediate	63	43	45	41	5/6[l]
Allyl	Allyl	6	40	Immediate	39	+	3	+	0/6
		6	50	Immediate	74	9	21	+	0/6
		6	60	Immediate	117	19	38	7	0/6
		6	70	Immediate	135	34	43	22	0/6
		6	80	Immediate	131	41	51	27	1/6
		6	90	Immediate	187	43	75	24	2/6
		6	100	Immediate	210	77	170	70	5/6
Propyl	Ethyl	10	20	0.5	39	+	+	+	0/10[m]
		10	40	Immediate	81	+	5	+	0/10[n]
		10	60	Immediate	90	9	18	1	0/10[n]
		10	80	Immediate	137	12	40	2	0/10[n]
		10	100	Immediate	210	44	60	18	3/10[n]
		10	120	Immediate	156	68	96	48	7/10[n]
Compound III R	**Compound III R1**								
Ethyl	Butyl	6	40	Immediate	12	+	1	+	0/6
		6	60	Immediate	14	+	9	+	0/6
		6	80	Immediate	26	5	15	3	0/6
		6	100	Immediate	43	23	30	15	0/6
		6	120	Immediate	53	29	31	24	2/6
Ethyl	Amyl	6	20	Immediate	6	+	+	+	0/6
		6	40	Immediate	16	+	6	+	0/6
		6	60	Immediate	26	11	13	+	0/6

[i] Labored respiration
[j] Drooling
[k] Constant chewing and drooling
[l] Scratching on awakening
[m] Tremors on awakening
[n] Tremors on awakening, salivation

(*Cont'd on next page*)

TABLE II.—EFFECTS OF INTRAVENOUS INJECTIONS IN WHITE RATS OF VARIOUS SUBSTITUTED BARBITURIC ACID DERIVATIVES AS HYPNOTICS AND THE OBSERVED TOXICITY. (*Cont'd from page 236*)

Compound III R	R₁	No. of Animals	Dose Mg./Kg.	Down After Injection, Min.	Duration of Loss of Various Reflexes, Min. Righting	Corneal	Ear	Tail	Killed
Ethyl	Amyl	6	80	Immediate	39	16	23	7	0/6
		6	100	Immediate	58	29	36	23	1/6
		6	120	Immediate	...	...	...	...	6/6
Allyl	Butyl	6	10	Immediate	4	+	+	+	0/6
		6	20	Immediate	12	2	8	+	0/6
		6	40	Immediate	20	6	11	+	0/6
		6	60	Immediate	24	11	13	2	0/6
		6	80	Immediate	51	26	27	23	3/6[m]
Isoamyl	Ethyl	6	60	Immediate	13	2	3	2	0/6[o]
		6	80	Immeditae	16	5	6	5	3/6[o]
Ethyl	1-Methyl-butyl	6	60	Immediate	13	+	+	+	0/6[p]
		6	80	Immediate	19	+	10	+	0/6[p]
		6	100	Immediate	25	2	13	+	0/6[p]
		6	120	Immediate	45	12	21	+	1/6[p]
		6	140	Immediate	38	11	24	10	4/6[p]
Isopropyl	Butyl	6	40	Immediate	15	+	5	+	0/6
		6	60	Immediate	20	9	13	+	0/6[p]
		6	80	Immediate	23	8	15	+	0/6[p]
		6	100	Immediate	31	19	20	13	1/6
		6	120	Immediate	37	25	31	23	4/6
Butyl	Allyl	6	80	Immediate	23	+	+	+	1/6[p]
		6	100	Immediate	20	2	4	+	4/6
Allyl	Ethyl	6	80	Immediate	40	+	9	+	0/6[q]
		6	100	Immediate	41	2	6	+	0/6[q]
		6	120	Immediate	57	13	34	+	1/6[q]
		6	140	Immediate	115	67	80	46	1/6[q]
		6	160	Immediate	68	37	40	30	2/6[q]
Methyl	Butyl	1	20	Immediate	...	...	...	...	..[r]
		1	30	Immediate	...	...	...	...	..[s]
		5	40	Immediate	...	...	...	...	..[s]
		1	60	Immediate	...	...	...	...	..[s]
		1	80	Immediate	...	...	...	...	..[s]
Allyl	Propyl	1	20	Not down	...	...	...	...	0/1
		9	30	Immediate	6	+	+	+	0/9[t]
		10	50	Immediate	11	1	4	+	0/10[u]
		10	70	Immediate	16	3	9	+	0/10[v]
		10	90	Immediate	33	14	20	9	4/10[w]
		10	110	Immediate	35	19	23	10	4/10[w]
Allyl	Isopropyl	1	20	Immediate	...	...	...	...	0/1[r]
		1	40	Immediate	...	...	...	...	0/1[r]
		1	60	.....	...	...	...	...	0/1[x]
Phenyl	Ethyl	10	40	Immediate	12	+	+	+	0/10[y]
		10	60	Immediate	28	+	2	+	0/10[z]
		10	80	Immediate	44	5	7	3	1/10[z]
		10	100	Immediate	59	6	9	4	0/10[aa]
		10	120	Immediate	63	13	17	10	0/10[aa]
		8	140	Immediate	126	18	24	15	0/8[bb]
		10	150	Immediate	122	12	19	9	3/10[bb]
		10	160	Immediate	107	13	18	10	6/10[bb]
		10	170	Immediate	146	17	23	9	9/10[bb]

[o] Convulsions, labored respiration
[p] Slight tremors on awakening
[q] Rapid respiration after injection
[r] No effect
[s] Incoordination only
[t] Grinding teeth
[u] Convulsive jerk on awakening
[v] Jerking during injection
[w] Shakes head on awakening
[x] Violent convulsions
[y] Conclusive twitch on awakening
[z] Labored respiration
[aa] Struggled during injection
[bb] Struggled during injection, lacrimation

TABLE III.—EFFECTS OF INTRAVENOUS INJECTIONS IN WHITE RATS OF VARIOUS SUBSTITUTED BARBITURIC ACID DERIVATIVES AS HYPNOTICS AND THE OBSERVED TOXICITY

Compound IV R	Compound IV R_1	No. of Animals	Dose, Mg./Kg.	Down After Injection, Min.	Duration of Loss of Various Reflexes, Min. Righting	Corneal	Ear	Tail	Killed
Ethyl	Butyl	6	30	Immediate	11	+	6	+	0/6
		6	40	Immediate	21	+	14	+	0/6
		6	50	Immediate	27	8	17	3	0/6
		6	60	Immediate	46	21	28	17	0/6
		6	70	Immediate	62	33	40	31	2/6
Allyl	Butyl	6	80	Immediate	66	34	39	30	4/6
		6	30	Immediate	7	+	3	+	0/6
		6	40	Immediate	14	+	8	+	0/6
		6	50	Immediate	18	4	11	+	0/6
		6	60	Immediate	25	9	16	5	1/6
		6	70	Immediate	...	...	...	...	6/6
		6	80	Immediate	20	10	15	+	5/6
Ethyl	Ethyl	6	80	Immediate	41	+	+	+	0/6[a]
		6	100	Immediate	84	+	18	+	0/6[a]
		6	120	Immediate	106	50	72	21	1/6[a]
		6	140	Immediate	117	64	80	36	5/6[a]
Allyl	Ethyl	6	80	Immediate	31	+	3	+	0/6[b]
		6	100	Immediate	33	18	29	+	1/6[c]
		6	120	Immediate	69	35	41	21	2/6[c]
		6	140	Immediate	80	40	64	20	4/6[c]
Compound V R	Compound V R_1								
Ethyl	Propyl	6	40	Immediate	9	+	+	+	0/6[d]
		6	60	Immediate	14	+	3	+	0/6
		6	80	Immediate	21	3	11	+	0/6
		6	100	Immediate	29	12	20	4	1/6[e]
		6	120	Immediate	35	16	21	4	0/6[e]
		6	140	Immediate	34	20	25	12	3/6
Evipal Sodium		10	30	Immediate	34	+	5	+	0/10[f]
		10	40	Immediate	28	+	6	+	0/10
		9	50	Immediate	40	1	9	+	0/9[g]
		6	60	Immediate	28	5	9	+	0/6
		6	70	Immediate	34	6	15	+	0/6
		6	80	Immediate	58	13	30	8	0/6
		6	100	Immediate	35	19	55	13	0/6
2% Solution		10	110	Immediate	53	9	19	5	1/10
5% Solution		10	110	Immediate	46	13	22	6	5/10
		10	120	Immediate	88	19	58	11	2/10
		10	130	Immediate	54	10	22	7	5/10
		6	140	Immediate	104	49	77	48	3/6
		6	160	Immediate	...	...	...	...	6/6
Amytal Sodium		6	20	Not Down	...	...	...	...	0/6
		6	40	1	92	+	+	+	0/6
		6	60	Immediate	128	2	18	+	0/6
		6	80	Immediate	192	38	85	+	1/6
		6	100	Immediate	248	76	143	+	0/6
		6	120	Immediate	292	107	176	81	1/6
		6	140	Immediate	340	180	255	98	4/6
		6	160	Immediate	428	294	330	285	4/6
Pentobarbital Sodium		6	20	Immediate	62	+	5	+	0/6
		6	30	Immediate	117	+	50	+	0/6
		6	40	Immediate	127	32	57	20	0/6
		6	60	Immediate	232	116	190	87	1/6
		6	80	Immediate	429	377	411	356	1/6
		6	100	Immediate	414	343	389	335	4/6

[a] Constant scratching, lacrimation
[b] Scratching
[c] Scratching, drooling
[d] Muscle tremors on awakening
[e] Tremors on awakening
[f] Grinding teeth—convulsions in 3
[g] Grinding teeth—1 animal

TABLE IV.—EFFECTS OF INTRAVENOUS INJECTIONS IN WHITE RATS OF VARIOUS SUBSTITUTED THIOBARBITURIC ACID DERIVATIVES AS HYPNOTICS AND THE OBSERVED TOXICITY

Compound VI R	R_1	No. of Animals Used	Dose, Mg./Kg.	Down After Injection, Min.	Duration of Loss of Various Reflexes, Min. Righting	Corneal	Ear	Tail	Killed
Secondary Butyl	Ethyl	6	40	Immediate	4	+	+	+	0/6
		6	60	Immediate	14	+	5	+	0/6
		6	100	Immediate	67	+	17	+	0/6[a]
		6	120	Immediate	102	4	12	+	1/6[a]
		6	140	Immediate	135	+	22	+	0/6[a]
		6	160	Immediate	203	44	55	2	0/6[a]
		6	200	Immediate	247	17	92	57	0/6[a]
		6	240	Immediate	329	65	145	25	2/6[b]
		6	280	Immediate	480	63	250	63	5/6[b]
1-Methyl-Butyl	Ethyl	6	20	Immediate	13	+	+	+	0/6
		6	40	Immediate	32	+	9	+	0/6
		6	60	Immediate	45	3	25	7	0/6
		5	80	Immediate	55	32	31	32	0/5
		6	100	Immediate	64	51	51	51	5/6
1-Methyl-Butyl	Allyl	6	80	Immediate	40	+	19	+	0/6[e]
Compound VII R	R_1								
Ethyl	Butyl	6	20	Immediate	6	+	+	+	0/6
		6	30	Immediate	10	+	4	+	0/6
		6	40	Immediate	20	6	11	+	0/6
		6	50	Immediate	24	1	11	+	0/6
		6	60	Immediate	30	9	17	+	0/6
		6	80	Immediate	115	25	34	6	1/6
		6	100	Immediate	130	34	53	16	5/6
Ethyl	Amyl	6	20	Immediate	8	+	1	+	0/6
		6	40	Immediate	23	4	13	4	0/6
		6	60	Immediate	34	15	22	8	0/6
		6	80	Immediate	113	25	45	23	1/6
		6	100	Immediate	86	3	38	13	1/6
		6	120	Immediate	...	...	...	...	6/6
Ethyl	Ethyl	6	40	Immediate	53	+	+	+	0/6
		6	60	Immediate	177	5	10	2	0/6
		6	70	Immediate	70	12	20	9	0/6
		6	80	Immediate	200	14	30	6	1/6
		6	90	Immediate	120	9	21	5	0/6
		12	100	Immediate	211	31	71	18	4/12
		6	110	Immediate	115	29	47	25	4/6
Allyl	Methyl	6	40	Immediate	48	3	10	+	0/6[d]
		6	60	Immediate	141	43	50	37	0/6
		6	80	Immediate	121	37	51	35	1/6
		6	100	Immediate	172	70	92	54	1/6
		6	120	Immediate	245	106	135	101	3/6
Amyl	Methyl	6	80	Immediate	32	+	12	+	0/6[e]
Allyl	Ethyl	6	30	Immediate	14	+	+	+	0/6
		6	40	Immediate	23	+	6	+	0/6
		6	50	Immediate	48	+	13	+	0/6
		6	60	Immediate	67	9	28	3	0/6
		6	70	Immediate	62	24	31	6	2/6
		6	80	Immediate	104	36	45	14	4/6
Propyl	Ethyl	10	20	Immediate	2	+	+	+	0/10[f]
		10	40	Immediate	24	+	3	+	0/10[g]
		10	60	Immediate	70	2	12	+	0/10[h]
Compound VIII R	R_1								
Ethyl	1-Methyl-Butyl	6	80	Immediate	24	+	13	+	0/6[i]
		6	100	Immediate	43	+	20	+	0/6[i]
Ethyl	Amyl	6	20	Immediate	2	+	+	+	0/6
		6	40	Immediate	12	+	7	+	0/6
		6	60	Immediate	21	+	11	+	0/6

[a] Muscle tremors after injection
[b] Hiccough respiration
[c] Convulsions during injection and for 3 min. Violent tremors on awakening
[d] Sneezing, shaking head on awakening
[e] Convulsions after injection
[f] Licking
[g] Convulsive twitching
[h] Convulsive twitching, shaking
[i] Muscle tremors

(Cont'd on next page)

TABLE IV.—EFFECTS OF INTRAVENOUS INJECTIONS IN WHITE RATS OF VARIOUS SUBSTITUTED THIOBARBITURIC ACID DERIVATIVES AS HYPNOTICS AND THE OBSERVED TOXICITY. (*Cont'd from page 239*)

Compound VIII R	R_1	No. of Animals Used	Dose Mg./Kg.	Down After Injection, Min.	Duration of Loss of Various Reflexes, Min. Righting	Corneal	Ear	Tail	Killed
Ethyl	Amyl	6	80	Immediate	35	9	21	+	0/6
		6	100	Immediate	62	27	41	8	0/6[j]
		6	120	Immediate	63	20	43	13	1/6
		6	140	Immediate	74	31	59	25	3/6
Ethyl	Butyl	6	20	Immediate	3	+	+	+	0/6
		6	40	Immediate	9	+	3	+	0/6
		6	60	Immediate	30	3	12	+	0/6
		6	80	Immediate	50	14	17	+	0/6
		6	100	Immediate	58	23	36	20	0/6[j]
		6	120	Immediate	100	40	72	14	1/6
		6	140	Immediate	107	54	65	41	3/6
Allyl	Butyl	10	15	Immediate	5	+	+	+	0/10
		10	20	Immediate	6	+	3	+	0/10
		10	25	Immediate	12	+	+	+	0/10
		12	40	Immediate	20	1	9	1	0/12
		12	60	Immediate	24	8	15	5	0/12
		12	80	Immediate	32	15	21	10	0/12
		22	100	Immediate	44	19	26	13	22/2
		10	110	Immediate	37	12	19	7	0/10
		10	120	Immediate	53	19	30	7	5/10
		10	130	Immediate	54	14	28	10	7/10
Butyl	Allyl	2	80	Immediate	13	+	+	+	0/2[k]
Isopropyl	Butyl	6	20	Immediate	8	+	2	+	0/6[l]
		6	40	Immediate	21	+	11	+	0/6[l]
		6	60	Immediate	35	2	16	+	0/6[l]
		6	80	Immediate	31	11	17	5	0/6[l]
		6	100	Immediate	48	20	26	7	1/6[l]
		6	120	Immediate	61	25	39	16	2/6[l]
Isoamyl	Ethyl	2	80	Not down	...	...	...	...	0/2[m]
Allyl	Ethyl	6	80	Immediate	33	+	8	+	0/6[n]
Compound IX R	R_1								
Ethyl	Butyl	6	10	Immediate	11	+	3	+	0/6
		6	20	Immediate	29	9	14	+	0/6
		6	40	Immediate	107	12	51	+	1/6
		6	60	Immediate	109	31	104	16	2/6
		6	80	Immediate	...	...	...	...	6/6
Compound X R	R_1								
Ethyl	Ethyl	6	40	Immediate	110	37	55	8	1/6[o]
		6	60	Immediate	192	130	122	39	3/6[p]
		6	80	Immediate	...	...	...	...	6/6
Compound XI R	R_1								
Ethyl	Ethyl	6	30	Immediate	20	+	3	+	0/6[q]
		6	40	Immediate	78	+	26	+	0/6[r]
		6	50	Immediate	119	9	28	+	1/6[s]
		6	60	Immediate	186	33	61	7	3/6
		6	70	Immediate	...	...	...	...	6/6
		6	80	Immediate	269	52	168	50	5/6
Allyl	Butyl	6	30	Immediate	18	+	6	+	0/6
		6	40	Immediate	21	+	11	+	0/6
		6	50	Immediate	31	+	11	+	0/6
		6	60	Immediate	33	5	15	3	0/6
		6	70	Immediate	...	...	...	...	6/6
Ethyl	Butyl	6	20	Immediate	19	+	5	+	0/6
		6	30	Immediate	62	10	31	3	1/6

[j] Tremors on awakening
[k] Violent convulsion during and after injection
[l] Slight tremors on awakening
[m] Violent convulsion during and after injection, strychnine-like
[n] Convulsions after injection, labored respiration
[o] Convulsive respiration
[p] Convulsive movements on awakening
[q] Convulsion
[r] Jerking and drooling
[s] Convulsion and drooling

(*Cont'd on next page*)

TABLE IV.—EFFECTS OF INTRAVENOUS INJECTIONS IN WHITE RATS OF VARIOUS SUBSTITUTED THIOBARBITURIC ACID DERIVATIVES AS HYPNOTICS AND THE OBSERVED TOXICITY. (*Cont'd from page 240*)

Compound XI R	Compound XI R_1	No. of Animals Used	Dose Mg./Kg.	Down After Injection, Min.	Duration of Loss of Various Reflexes, Min. Righting	Corneal	Ear	Tail	Killed
Ethyl	Butyl	6	40	Immediate	73	29	44	14	0/6
		6	50	Immediate	...	...	...	...	6/6
		6	60	Immediate	...	...	...	...	6/6
Allyl	Ethyl	6	40	Immediate	21	+	1	+	0/6[t]
		6	50	Immediate	23	+	8	+	0/6[t]
		6	60	Immediate	37	3	17	+	0/6[t]
		6	70	Immediate	42	4	19	+	0/6[t]
		6	80	Immediate	54	10	30	3	1/6[t]
		6	100	Immediate	66	11	32	6	3/6[t]
Pentothal Sodium		10	15	Immediate	27	+	2	+	0/10[u]
		10	20	Immediate	39	+	11	+	0/10
		10	25	Immediate	41	+	9	+	0/10[v]
		12	40	Immediate	226	41	113	27	1/12
		10	50	Immediate	312	55	202	33	6/10
		22	60	Immediate	366	118	253	65	17/22
		6	80	Immediate	...	...	...	...	6/6

[t] Convulsions on injection and awakening

[u] Shakes head, moves lower jaw

[v] Respiratory convulsions

TABLE V.—OBSERVED TOXICITY IN WHITE RATS FOLLOWING ORAL ADMINISTRATION OF VARIOUS SUBSTITUTED BARBITURIC AND THIOBARBITURIC ACID DERIVATIVES

Compound	R	R_1	LD_{50}
I	1-Methylbutyl	Ethyl	366
	Isobutyl	Methyl	505
	1-Methylbutyl	Allyl	702
	1-Methylbutyl	Methyl	364
II	Ethyl	Butyl	582
	Allyl	Butyl	639
	Isobutyl	Allyl	417
	Allyl	Ethyl	356
	Allyl	Allyl	346
VII	Ethyl	Ethyl	772
VIII	Allyl	Butyl	1453
Pentobarbital Sodium			99

Chronic Toxicity—Rat.—Seven of the new derivatives were administered orally to rats in chronic studies. Doses of the compounds representing 25% of the LD_{50} were given twice daily for fourteen consecutive days and the animals then sacrificed for pathological examination. Fifteen animals were dosed in this manner for each of the compounds investigated. The pathologic findings, summarized in Table VI, appear to indicate the absence of gross or irreversible changes in the tissues and organs examined.

Duration of Effect and Toxicity—Mouse.—In this study male, white, Swiss-strain mice, weighing between 20 and 35 Gm., were injected intraperitoneally with selected doses of the sodium barbiturates in aqueous solution. The values shown in the tables are, however, calculated as quantities of free acid. Sodium Amytal, Sodium Pentobarbital, and Sodium Evipal were used as comparative test agents. The observations recorded in Table VII include (*a*) the mean hypnotic dose, (*b*) duration of sleep as indicated by the loss and reappearance of the righting reflex, (*c*) the time required to regain normal movement, and (*d*) the acute toxicity.

The mean hypnotic dose, defined as the dose producing brief loss of righting reflex in 50% of the animals, was determined by injecting groups of 10 animals with 0.5% to 1.0% solutions at 3 dosage levels. Data for the estimation of the acute toxicities were obtained by injecting, at 5 to 10 predetermined dosage levels, 2% solutions of the sodium barbiturates. With the results thus obtained values of the LD_{50} were computed by the use of Kärber's method. Having established the LD_{50} for each compound, it was possible to compare the effects of the barbiturates by injecting in each instance a single dose equivalent to 60% of the LD_{50} (see Table VII). For this purpose 10 animals were injected with each compound.

The variation in duration of action and toxicity among the barbiturates observed in the mouse was comparable to that seen in the rat.

TABLE VI.—EFFECTS OF CHRONIC ORAL ADMINISTRATION IN WHITE RATS OF VARIOUS SUBSTITUTED BARBITURIC AND THIOBARBITURIC ACID DERIVATIVES

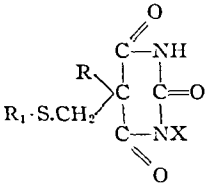
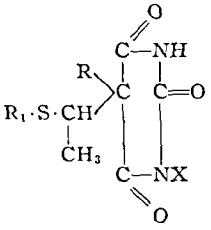
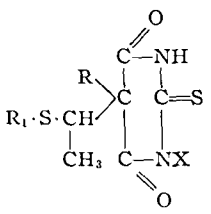
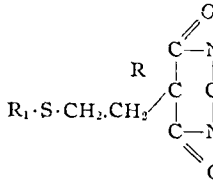
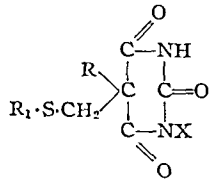
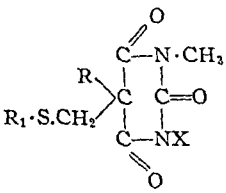
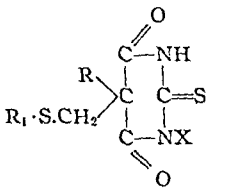
Compound	R	R_1	No. of Animals	Dose, 25% LD_{50}, Mg./Kg.	Consecutive Days Dosed	Pathologic Findings
	1-Methylbutyl	Allyl	15	176	14	Acute glomerulonephritis Fatty metamorphosis of liver
	Ethyl	Butyl	15	146	14	Normal
	Allyl	Butyl	15	160	14	Very mild acute glomerulonephritis Very mild metamorphosis of liver
	Isobutyl	Allyl	15	104	14	Normal
	Allyl	Ethyl	15	89	14	Normal
	Ethyl	Ethyl	15	193	14	One kidney showed localized inflammatory lesion in cortex Lesion in healing stage and of many days duration
	Allyl	Butyl	15	363	14	Very mild acute glomerulonephritis Mild fatty metamorphosis of liver

TABLE VII.—EFFECTS OF INTRAPERITONEAL INJECTIONS IN WHITE MICE OF VARIOUS SUBSTITUTED BARBITURIC AND THIOBARBITURIC ACID DERIVATIVES AS HYPNOTICS AND THE OBSERVED TOXICITY

Compound	R	R_1	*MHD*, Mg./Kg.	LD_{50}, Mg./Kg.	Duration of Anesthesia, 60% LD_{50}, Min.	Incoordination, 60% LD_{50}, Min.
	Methyl	Ethyl	175	750	330	0
	Methyl	Butyl	90	379	150	13
	Isobutyl	Methyl	65	354	108	24
	Allyl	Propyl	30	284	117	12
	Allyl	Butyl	20	194	123	14
	Isopropyl	Butyl	30	252	38	10
	Butyl	Butyl	30	268	95	20
	Isobutyl	Butyl	40	300	78	16
	Secondary Butyl	Butyl	40	244	69	30
	Ethyl	Ethyl	75	480	143	13
	Ethyl	Propyl	60	318	59	13
	Ethyl	Isopropyl	85	415	139	14
	Ethyl	Butyl	35	213	66	7
	Ethyl	Amyl	20	191	75	19
	Propyl	Ethyl	50	397	150	12
	Isopropyl	Ethyl	75	286	86	13
	Allyl	Ethyl	60	380	164	14
	Butyl	Ethyl	85	379	74	10
	Isobutyl	Ethyl	50	335	81	8

(*Cont'd on next page*)

TABLE VII.—EFFECTS OF INTRAPERITONEAL INJECTIONS IN WHITE MICE OF VARIOUS SUBSTITUTED BARBITURIC AND THIOBARBITURIC ACID DERIVATIVES AS HYPNOTICS AND THE OBSERVED TOXICITY
(*Cont'd from page 242*)

Compound	R	R_1	*MHD* Mg./Kg.	LD_{50} Mg./Kg.	Duration of Anesthesia 60% LD_{50}, Min.	Incoordination 60% LD_{50}, Min.
	Secondary Butyl	Ethyl	40	270	61	11
	1-Methylbutyl	Ethyl	45	213	35	8
	Isoamyl	Ethyl	90	285	8	6
	Propyl	Butyl	30	272	76	15
	Ethyl	Secondary Butyl	...	231	...	..
	Ethyl	Isobutyl	...	277	...	..
	Methallyl	Ethyl	...	250	...	..
	Ethyl	Tertiary Butyl	...	256	...	..
	Methyl	Ethyl	...	446	166	12
	Methyl	Butyl	50	305	116	13
	Propyl	Ethyl	...	541	168	30
	Isobutyl	Ethyl	...	420	Convulsive	..
	Ethyl	Ethyl	...	350	Convulsive	..
	Isopropyl	Ethyl	75	350	Convulsive	..
	Ethyl	Isopropyl	...	100–200	...	..
	Ethyl	Propyl	75	239	41	13
	Ethyl	Ethyl	...	402	190	21
	Secondary Butyl	Ethyl	75	375	...	..
	1-Methylbutyl	Ethyl	75	350	...	..
	Isobutyl	Methyl	75	314	Convulsive	..
	Isobutyl	Ethyl	75	360	...	..
Evipal Sodium	....	....	...	220	35	16
Pentobaribtal Sodium	....	....	...	155	120	7
Amytal Sodium	....	....	...	260	99	7

SUMMARY

1. A group of new barbituric and thiobarbituric acids have been examined experimentally by intravenous injection in the rat and intraperitoneal injection in the mouse.

2. Data have been compiled on (*a*) the time of onset of hypnosis, (*b*) duration of action as revealed by various criteria, and (*c*) toxicity.

3. Several of the new derivatives have been administered orally to rats and the tolerance to repeated doses determined.

4. The observations permit the conclusion that the duration of anesthetic or hypnotic effect of the new compounds decreased as the molecular weight increased. Generally the series containing R'SCH— as

$$\begin{array}{c} R'SCH- \\ | \\ CH_3 \end{array}$$

a substituent were more active compounds as revealed by the acute toxicity. In an extensive series of compounds as described in this paper there are notable exceptions to any generalization regarding the relationship of chemical structure to activity.

REFERENCES

(1) Walter, L. A., Goodson, L. H., and Fosbinder, R. J., *J. Am. Chem. Soc.*, 67, 655, 657, 659, 661(1945).
(2) Barlow, O. W., Climenko, D. R., and Homburger, E., *Proc. Soc. Exptl. Biol. Med.*, 49, 11(1942).
(3) Kinsey, V. Everett, This Jo
(4) Kärber, *Arch. Expt. Path.* u
through Burn, J. H., "Biological
Univ. Press, London, 1937.

The Anesthetic Effects and Toxicities of Some New Barbituric Acid Derivatives Administered to Rabbits. II*

By WILLIAM H. HUNT† RUSSEL J. FOSBINDER, and O. W. BARLOW

IN A PREVIOUS paper (1) we have described the results obtained with a new series of barbituric acid derivatives following intravenous and intraperitoneal administration to the rat and mouse, respectively.

Continuing the experimental study of the new compounds reported by Walter, Goodson, and Fosbinder (2) we present herein pertinent data relative to the hypnotic action and toxicities of the barbituric acid derivatives administered intravenously to rabbits. Observations on the rate of detoxification of selected compounds are also included.

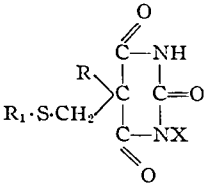
* Received Dec. 17, 1945, from the Research Laboratories of the Maltbie Chemical Co., Newark, N. J., and the Winthrop Chemical Co., Rensselaer, N. Y.

† Present address: Marvin R. Thompson Laboratories, Stamford, Conn.

EXPERIMENTAL

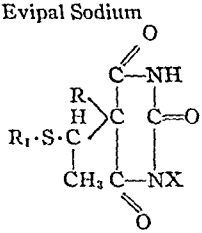
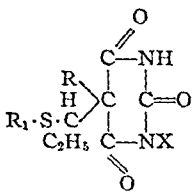
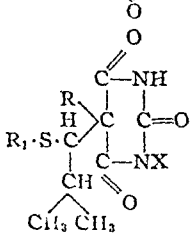
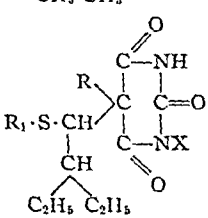
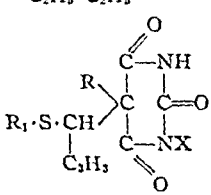
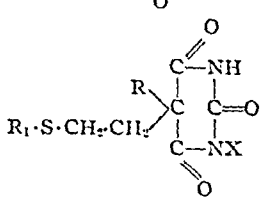
Duration of Effect and Toxicity.—Adult male, white, New Zealand-strain rabbits, averaging 2.5 Kg. in weight, were employed in this study. Five

TABLE I.—DURATION OF HYPNOTIC EFFECTS AND OBSERVED TOXICITIES OF VARIOUS SUBSTITUTED BARBITURIC ACID DERIVATIVES INJECTED INTRAVENOUSLY IN RABBITS

Compound	R	R_1	MHD_{50}, Mg./Kg.	LD_{50}, Mg./Kg.	Duration Anesthesia, 60% LD_{50}, Min.	Incoordination, 60% LD_{50}, Min.	Remarks
	Allyl	Propyl	25	73	37	66	
	Allyl	Butyl	10	47	39	24	
	Isopropyl	Butyl	15	53	43	28	
	Butyl	Butyl	20	60	30	22	
	Isobutyl	Butyl	20	62	25	31	
	Secondary Butyl	Butyl	10	44	27	26	
	Ethyl	Ethyl	50	211	121	74	
	Ethyl	Propyl	40	124	33	51	
	Ethyl	Butyl	25	78	36	26	
	Ethyl	Isobutyl	40	104	39	37	Locomotion of forelegs
	Ethyl	Tertiary Butyl	..	162	...	..	Locomotion of forelegs
	Ethyl	Amyl	15	53	25	19	
	Ethyl	1-Methylbutyl	25	73	62	11	Locomotion of forelegs
	Ethyl	Isoamyl	20	61	37	12	
	Ethyl	Hexyl	15	43	15	13	
	Ethyl	2-Ethylbutyl	20	65	22	14	
	Ethyl	Cyclohexyl	..	...	...	..	Violent convulsions
	Propyl	Ethyl	40	171	120	97	
	Isopropyl	Ethyl	30	106	92	45	Sneezing on awakening
	Allyl	Ethyl	30	158	106	28	
	Isobutyl	Ethyl	35	115	72	46	
	Secondary Butyl	Ethyl	27	101	67	44	
	1-Methylbutyl	Ethyl	22	70	37	20	Locomotion of forelegs
	Isoamyl	Ethyl	43	124	28	16	
	Cyclohexenyl	Ethyl	30	93	59	38	Locomotion of legs
	Hexyl	Ethyl	20	44	Very brief		
	Ethyl	Allyl	45	156	117	41	Locomotion of legs, convulsive action
	Methallyl	Ethyl	..	...	...	..	Locomotion of legs, convulsive action
	Propyl	Butyl	20	69	48	39	

(Cont'd on next page)

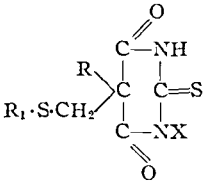
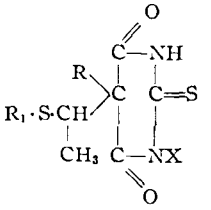
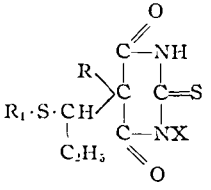
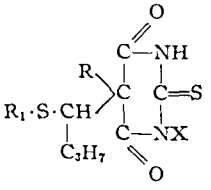
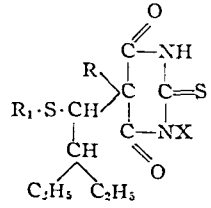
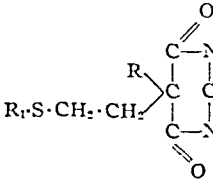
TABLE I.—DURATION OF HYPNOTIC EFFECTS AND OBSERVED TOXICITIES OF VARIOUS SUBSTITUTED BARBITURIC ACID DERIVATIVES INJECTED INTRAVENOUSLY IN RABBITS. (*Cont'd from page 244*)

Compound	R	R_1	MHD_{50}, Mg./Kg.	LD_{50}, Mg./Kg.	Duration Anesthesia, 60% LD_{50}, Min.	Incoordination, 60% LD_{50}, Min.	Remarks
Pentobarbital Sodium			..	45	58	24	
Amytal Sodium			..	75	89	48	
Evipal Sodium			15	80	19	13	
	Propyl	Isobutyl	35	116	45	19	Convulsive tremors
	Isobutyl	Allyl	..	46	28[a]	..	Locomotion of legs, convulsive action
	Allyl	Butyl	10	35	34	21	
	Ethyl	Butyl	15	55	34	21	Locomotion of legs, convulsive twitch
	Propyl	Butyl	20	58	35	32	Locomotion of legs
	Ethyl	Amyl	10	36	28	9	
	Ethyl	Isoamyl	15	45	41	10	Locomotion of legs, convulsive action
	Hexyl	Ethyl	20	45	12	17	Locomotion of legs
	Methyl	Cyclohexyl	..	...	...	..	Convulsions and nystagmus
	Propyl	Methyl	..	...	...	..	M. H. D. between 61–124 mg./Kg.
	Ethyl	Butyl	20	65	52	21	Convulsive action
	Ethyl	Isopropyl	..	10	...	..	Strychnine-like death
	Ethyl	Ethyl	30	44	12	14	
	Ethyl	Ethyl	54	65	...	..	
	Ethyl	Ethyl	..	58	...	..	Convulsive action
	Ethyl	Butyl	25	73	24	8	

a Dose, 50% LD_{50}.

and 10% solutions of the sodium barbiturates were used throughout the experiments, permitting the administration of fairly uniform volumes in each instance. The injections were made into the marginal ear vein at a constant rate of 2.0 cc.. per minute. In this study, Sodium Pentobarbital, Sodium Pentothal, and Sodium Evipal were used as reference compounds. The criteria employed for the determination of the mean hypnotic dose, duration of anesthesia, and the incoordination interval were roughly similar to those used by Werner, Pratt, and Tatum (3). The acute toxicities of the barbiturates

TABLE II.—DURATION OF HYPNOTIC EFFECTS AND OBSERVED TOXICITIES OF VARIOUS SUBSTITUTED THIOBARBITURIC ACID DERIVATIVES INJECTED INTRAVENOUSLY IN RABBITS

Compound	R	R_1	MHD_{50}, Mg./Kg.	LD_{50}, Mg./Kg.	Duration Anesthesia, 60% LD_{50}, Min.	Incoordination, 60%. LD_{50}, Min.	Remarks
	Isobutyl	Methyl	.	110	50	40	
	Ethyl	Isoamyl	20	61	12	5	Very irritant on injection
	Isobutyl	Ethyl	30	129	45	39	
	Secondary Butyl	Ethyl	25	82	48	23	
	1-Methyl-butyl	Ethyl	20	56	16	12	
	Butyl	Butyl	..	...	..	..	Convulsions
	Propyl	Methyl	..	103	..	..	Very long duration
	Hexyl	Ethyl	25	58	8	4	Convulsions
	Ethyl	Butyl	15	57	23	16	Locomotion of legs
	Propyl	Butyl	20	58	21	21	Convulsive twitches
	Ethyl	Amyl	15	45	14	4	
	Ethyl	Isoamyl	20	51	15	6	
	Methyl	Cyclohexyl	..	...	..	..	Violent Convulsions, nystagmus
	Allyl	Butyl	15	40	22	11	Locomotion of legs
	Isobutyl	Allyl	20	73	30	33	Convulsive irritant on injection
	Ethyl	Isopropyl	..	...	..	..	Strychnine-like death
	Ethyl	Butyl	15	40	18	9	
	Ethyl	Ethyl	10	32	9	4	Convulsive action
	Ethyl	Ethyl	..	...	..	..	Cyanosis, mercaptan breath, appears to produce methemoglobin
	Allyl	Butyl	20	60	..	..	

were estimated by the application of Behrens' method (4). For the determination of the MHD_{50} (median hypnotic dose) and the LD_{50}, 10 animals were injected at each dosage level. Those animals used more than once were given a rest period sufficient for the clearance of residual barbiturate. A direct comparison of the duration of anesthesia produced by the new barbiturates and the reference compounds was obtained by injecting groups of 10 animals with a dose corresponding to 60% of the estimated LD_{50}.

The results are summarized in Tables I and II wherein the various compounds are listed and data are given on the MHD_{50}, the LD_{50}, duration of anesthesia produced by injection of a dose equivalent to 60 per cent of the LD_{50}, and the incoordination time at this dosage level. Side-effects observed following intravenous administration are included under the heading "Remarks." Of particular interest was the convulsant action of 5-Ethyl,5-α-isopropylthiopropyl barbiturate sodium which is structurally related to Sodium 5-ethyl,5-1,3-dimethylbutyl barbiturate studied by Swanson and Chen (5) who reported the barbiturate was a convulsant in

TABLE III.—DETOXIFICATION OF VARIOUS SUBSTITUTED BARBITURIC AND THIOBARBITURIC ACID DERIVATIVES ADMINISTERED INTRAVENOUSLY TO RABBITS

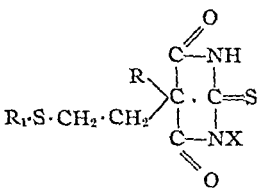
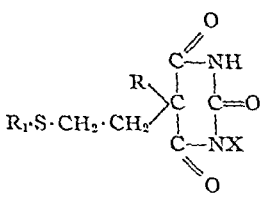
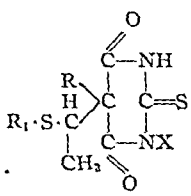
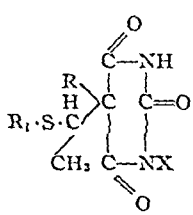
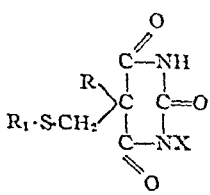
Compound	R	R_1	No. of Animals	Single Dose, Mg./Kg.	Per Cent, LD_{50}	Time Interval, Min.	No. of Doses	Average	Total Dose, % LD_{50}	Average, % LD_{50}
Evipal Sodium			10	27	33	15	4–7	5.1	135–236	172.3
			10	40	50	15	1–4	2.9	50–200	145.0
			5	40	50	23	2–4	3.0	100–200	150.0
Pentothal Sodium			10	13	33	15	5–10	7.1	138–325	228.5
			10	20	50	15	3–5	4.1	150–250	205.0
			5	20	50	23	3–5	3.8	150–250	190.0
	Allyl	Butyl	10	20	33	15	9–17	12.7	300–567	380.3
			10	30	50	15	3–11	7.5	150–550	375.0
			5	30	50	23	10	10.0	No Deaths	
	Allyl	Butyl	5	30	50	15	3–5	3.4	150–250	170.0
	Isopropyl	Butyl	5	30	50	15	3–5	3.6	150–250	180.0
	Ethyl	Butyl	5	37	50	23	3–4	3.4	150–200	170.0
	Ethyl	Amyl	5	30	50	15	3–5	3.8	150–250	190.0
	Ethyl	Ethyl	5	17	33	15	7–12	9.6	238–408	326.0
			5	25	50	15	4–6	5.2	200–300	260.0
			5	25	50	23	5–8	6.6	250–400	330.0
	Isobutyl	Allyl	10	16	33	15	4–9	6.8	133–313	231.2
			10	24	50	15	2–5	3.8	100–250	190.0
			5	24	50	23	3–4	4.2	150–200	180.0
	Isopropyl	Butyl	5	27	50	15	3–4	3.6	150–200	180.0
	Secondary Butyl	Butyl	5	22	50	15	4–9	6.0	200–450	300.0

TABLE IV.—DETOXIFICATION OF VARIOUS SUBSTITUTED BARBITURIC AND THIOBARBITURIC ACID DERIVATIVES ADMINISTERED INTRAVENOUSLY TO RABBITS

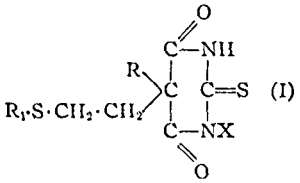
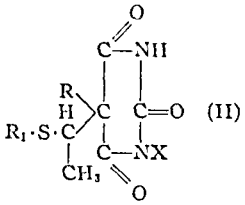
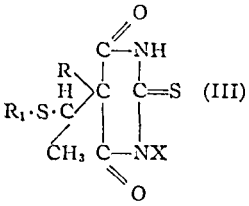
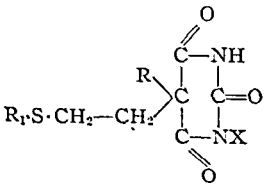
Compound	R	R_1	No. of Animals	Dose Level	No. of Animals Dead After 15-Min Intervals — No. of Intervals 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	Survivors
Evipal Sodium			10	33% LD_{50}	..	..	.	3	4	2	1					..	..	..	..	..	..	
Pentothal Sodium			10	33% LD_{50}	..	..	..	..	1	2	4	2	..	1	.	..	..	..	..	..	..	
 (I)	Allyl	Butyl	10	33% LD_{50}	..	..	..	..	..	..	..	..	1	..	2	1	3	..	1	.	1	1
 (II)	Isobutyl	Allyl	10	33% LD_{50}	..	..	..	1	1	3	1	2	2	..	..	..	..	..	..	..	..	
 (III)	Ethyl	Ethyl	5	33% LD_{50}	..	..	..	..	..	..	1	1	2	..	..	2	..	..	..	..	..	
Evipal Sodium	.....	...	10	50% LD_{50}	1	..	8	1	..	..	...	..	..	..	..	..	..	..	..	..	..	
Pentothal Sodium	.....	...	10	50% LD_{50}	..	..	2	5	3	..	..	..	..	..	..	..	..	..	..	..	..	
(I)	Allyl	Butyl	10	50% LD_{50}	..	..	1	1	..	..	3	1	2	1	1	..	..	..	..	..	..	
(II)	Isobutyl	Allyl	10	50% LD_{50}	..	1	2	5	2	..	..	..	..	..	..	..	..	..	..	..	..	
(III)	Ethyl	Ethyl	5	50% LD_{50}	..	..	..	1	2	2	..	..	..	..	..	..	..	..	..	..	..	..
	Ethyl	Amyl	5	50% LD_{50}	..	..	2	2	1	..	..	..	..	..	..	..	..	..	..	..	..	
	Isopropyl	Butyl	5	50% LD_{50}	..	..	3	1	1	..	..	..	..	..	..	..	..	..	..	..	..	
	Allyl	Butyl	5	50% LD_{50}	..	..	4	..	1	..	..	..	..	..	..	..	..	..	..	..	..	
	Ethyl	Butyl	5	50% LD_{50}	..	..	..	3	1	1	..	..	..	..	..	..	..	..	..	..	..	
 (IV)																						

TABLE IV.—DETOXIFICATION OF VARIOUS SUBSTITUTED BARBITURIC AND THIOBARBITURIC ACID DERIVATIVES ADMINISTERED INTRAVENOUSLY TO RABBITS

(*Cont'd from page 248*)

Compound	R	R_1	No. of Animals	Dose Level	No. of Animals Dead After 15-Min. Intervals: 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	Survivors
Evipal Sodium	Isopropyl	Butyl	5	50% LD_{50}	..	..	2	3	..	..	..	..	1	..	..	..	..	..	..	..	..	
Pentothal Sodium	Secondary Butyl	Butyl	5	50% LD_{50}	..	..	..	1	1	2	..	..	..	..	..	..	..	..	..	..	..	
(I)			5[a]	50% LD_{50}	..	2	1	2	..	..	..	..	..	..	..	..	..	..	..	..	..	
(II)			5[a]	50% LD_{50}	..	..	2	2	1	..	..	..	..	..	..	..	..	..	..	..	..	
(III)			5[a]	50% LD_{50}	..	..	..	3	..	..	..	..	..	..	..	..	..	..	..	..	..	5
(IV)			5[a]	50% LD_{50}	..	..	2	..	..	..	..	..	..	..	..	..	..	..	..	..	..	
			5[a]	50% LD_{50}	..	..	3	2	..	..	..	..	..	..	..	..	..	..	..	..	..	

[a] Injected at 23-min. intervals.

warm-blooded animals when given in relatively low dosage. In this instance the substitution of —CH_2— by —S— apparently did not alter the pharmacological action.

All of the compounds appeared to depress the respiratory rate to a varying degree and in general the effect was dependent on the size of the dose administered. A pharmacodynamic study of this depression was not attempted.

As a part of a more detailed study of several barbiturates, selected from the group of compounds examined, the rate of a detoxification following intravenous administration was determined using a procedure similar to that described by Hazleton, Koppanyi, and Linegar (6); Pentothal Sodium and Evipal Sodium were used as control agents. In separate groups of animals, doses equivalent to 33% and 50% of the LD_{50} were given at fifteen-minute intervals until death ensued, while another group received doses of 50% of the LD_{50} at twenty-three-minute intervals. The data shown in Tables III and IV summarize the results thus obtained.

SUMMARY

1. A group of new barbituric and thiobarbituric acids have been studied by intravenous injection in rabbits. The median hypnotic dose, acute toxicities, and duration of action at a uniform dosage level, have been determined and described.

2. The rate of detoxification on intravenous administration has been determined for several of the compounds. Sodium 5-allyl-5-(α-butylthioethyl) thiobarbiturate and Sodium 5-ethyl-5-(α-ethylthioethyl) thiobarbiturate were rapidly detoxified by rabbits.

3. When the new barbiturates were administered intravenously it was observed that the duration of hypnotic effect decreased and the toxicity increased as the molecular weight of the compounds increased. Derivatives comprising the RSCH— series were generally more toxic

$$\begin{array}{l} \text{RSCH—} \\ \quad\ \ | \\ \quad\ \ CH_3 \end{array}$$

than those in the analogous $RSCH_2$— series.

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The Influence of Physiologically Active Compounds and Their Inactive Homologs on Phase Boundary Potentials*

By CHARLES P. DILLON,† GAYLORD B. ESTABROOK, and WALTER H. HARTUNG

Employing a "model cell," as described by Beutner, the change produced in electromotive force by propadrine and three homologs and by choline and two homologs was determined. The results show that it is not yet possible to correlate change in E. M. F. with physiological activity in homologous series; there is an approximate parallelism between effect on E. M. F. and relative toxicity.

BEUTNER (1) suggests that many drugs, particularly those which are active in small doses, produce their physiological effect by altering the electromotive forces which normally exist between the heterogeneous components of the protoplasm. He has elaborated on this hypothesis and produced experimental data in its support (2).

For example, a "model cell" was set up as follows:

Hg^-	KCl solution saturated with HgCl	Nitrobenzene and 10% oleic acid	Physiological saline with 0.02% sodium oleate. Drug added here	KCl saturated with HgCl	Hg^+

The normal electromotive force of this cell, without alkaloid or drug, is approximately 85 millivolts. The addition of drug or chemical to the physiological saline solution at the point indicated reduces the E. M. F. Typical results are assembled in Table I.

Acetylcholine at a concentration of $1:3 \times 10^{-4}$ produces a negative potential of 200 mv. and at $1:1 \times 10^{-8}$, a negative potential of 5 mg. (4). Paredrine at a concentration of $1:5 \times 10^{-4}$, in a cell using tricaproin as the oil phase, reduced the potential 26 mv. (5).

The implications of the hypothesis are manifold and intriguing. It will be observed, however, even when all the data are considered, that Beutner has confined his studies to compounds known to be physiologically active and generally useful. This raises the question as to whether or not these electrical properties are characteristic only for the active members of a homologous series of compounds. If this should prove true, then it is reasonable to expect that biological activity could be predicted for an unknown compound merely by measuring the change it produces in the E. M. F. of a model cell; the character of the physiological properties may then be determined by subsequent *in vivo* examination. If so, such measurements should provide an invaluable means by which the chemist and the pharmacologist, in their search for new drugs, could narrow their investigations by prompt elimination of unpromising compounds.

Two series, the active members of which have been studied by Beutner, are available, viz., the pressor amines derived structurally from β-phenylethylamine and the quaternary ammonium compounds related to choline.

TABLE I.—EFFECT OF ALKALOIDS AND THEIR SALTS ON E. M. F. OF "MODEL CELL" (3)

Substance	Concentration $\times 10^6$	Decrease in E. M. F., Mv.
Pilocarpine	1	5
	10	33
	50	57
Atropine sulfate	1	4
	10	25
	50	57
Strychnine sulfate	1	4
	5	18
	10	31
Quinine hydrochloride	1	1
	10	11
Morphine sulfate	2	6
	5	11
Ephedrine sulfate	1	6
Cocaine hydrochloride	1	3

An extensive series of compounds struc-

* Received April 29, 1946, from the Research Laboratories, School of Pharmacy, University of Maryland, Baltimore, Md.
† Present address: St. Joseph's College, Mountain View, Calif.

turally related to epinephrine, ephedrine, and benzedrine was available from previous studies in correlating the relationship between chemical structure and physiological activity (6).

A second group of related compounds is found in the choline series, members of which were placed at our disposal by Merck & Company, through the courtesy of Drs. Bastedo and Major. Choline and β-methylcholine are active, and the relative inertness and greater toxicity of "triethylcholine" are known (7).

It is obvious that any model cell can at best only approximate biological conditions, not duplicate them. Hence, negative results are not conclusive; rather they add complexity to the hypothesis. For instance, is it perhaps the products resulting from the reaction of the drug with the protoplasm which causes the electromotive variation? And physiologically inactive homologs or isomers do not give rise to products which produce a change in E. M. F.?

EXPERIMENTAL

The cell used in these experiments was similar to that described by Beutner (1). The electrodes were of the usual calomel type filled with saturated potassium chloride solution. To one of the electrodes was sealed a hook-shaped tube, at the lower end of which was fused a cup of 2- or 3-ml. capacity. This cup held the "oil" which set up the phase boundary potential.

For measuring the changes in potential difference a Beckman *p*H meter, laboratory model G, was used. This type of potential measuring apparatus was applicable, since the cell had very high internal resistance. This instrument gave measurements reproducible to 5 mv. Readings were taken with the poles connected one way, and then repeated with the poles reversed.

In making a run, potassium chloride solution was pipetted from the cup and the oil introduced, also by pipette. To replace the oil, it was first removed by pipette, then 1 to 2 ml. potassium chloride solution was run into the calomel electrode from the top to force out any boundary potassium chloride. This was withdrawn, in turn, from the cup, and a new sample of oil added.

The compound being studied was made up in 0.01, 0.001, and, in some cases, 0.0001 molar solutions, the solvent being a solution of potassium chloride, 0.756 Gm./liter, or sodium benzoate, 0.001 molar. The central beaker of the cell was filled with 100 ml. of this salt solution and stirred until the salt distribution between the oil and the water reached equilibrium, and the potential difference was then measured. Into this was pipetted an amount of drug solution necessary to give the desired concentration, and the potential difference was again observed. The difference between this and the initial readings represented the change in potential difference due to concentration. More of the drug solution was then added until the desired next higher concentration was reached. Since the same salt solution served both as zero reading and as solvent for the compound studied, the salt concentration always remained the same throughout a series of readings. The oils used were guaiacol and nitrobenzene, each freshly distilled.

The data are summarized graphically. Figure 1

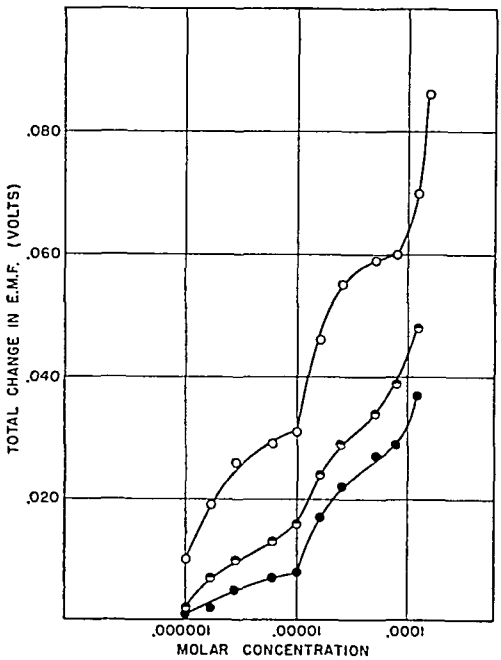

Figure 1.—○, propadrine HCl; ●, propadrine base O, hexadrine HCl.

shows the results obtained with propadrine base, its hydrochloride and hexadrine hydrochloride ($C_6H_5—CHOH—\underset{\underset{NH_2\cdot HCl}{|}}{CH}—C_4H_9$); nitrobenzene was used in the cup and sodium benzoate as the solution. Hexadrine hydrochloride gave, over a wide range of concentrations, a much greater change in voltage than the propadrine hydrochloride. It will be noted that the curves have approximately the same shape and slope. The differences in voltage correspond with the known toxicity of the compounds, the higher homolog being the more toxic (8). Propadrine and its salt produce approximately the same voltage change; this indicates an interchange of ions in the nitrobenzene.

Figure 3 shows the results obtained with propadrine, butadrine, and pentadrine (C_6H_5—CHOH—CH—R, R = CH_3, C_2H_5, and n-C_3H_7, respectively)
|
NH_2
hydrochlorides, using guaiacol and potassium chloride solution. Considered from the point of toxicity, the curves are in proper order, since pentadrine is about three times more toxic than propadrine (8).

Figure 2 shows the results obtained with choline chloride, methylcholine chloride and "triethylcholine" chloride, $HOCH_2CH_2$—$N(C_2H_5)_3Cl$, with sodium benzoate as the solution and nitrobenzene as the oil. Again, the change in voltage gives no indication of physiological activity, but the compound showing the greatest change in voltage is the most toxic.

In Fig. 3 are shown also the results obtained with cocaine hydrochloride and papaverine hydrochloride, the cell combination being guaiacol and potassium chloride. Here again, the more toxic cocaine produces the greater change in voltage.

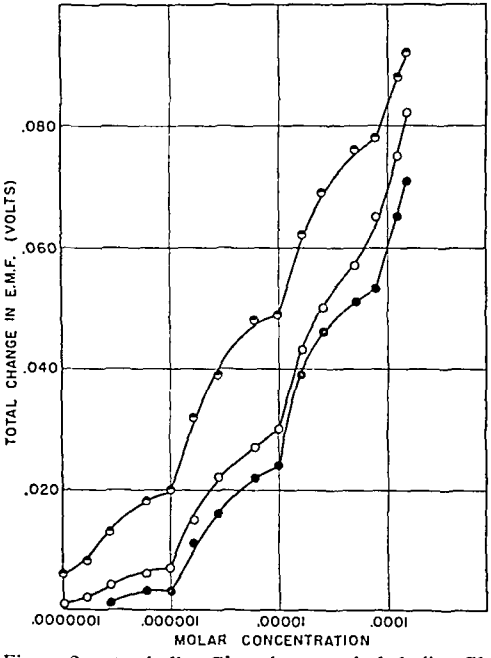

Figure 2.—●, choline Cl; ○, beta methyl choline Cl; ◓, triethyl choline Cl.

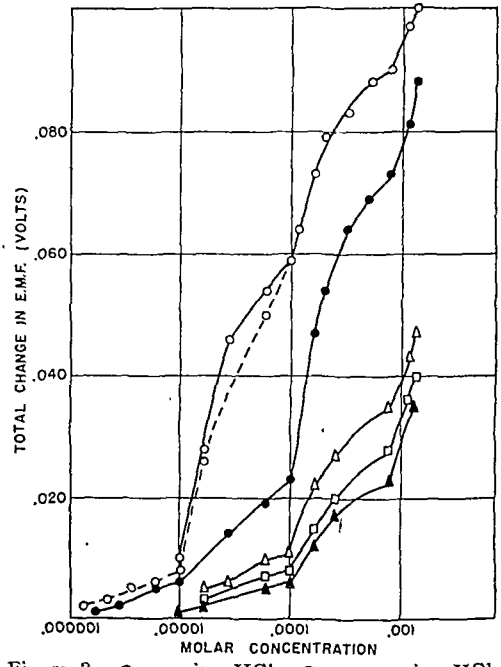

Figure 3.—○, cocaine HCl; ●, papaverine HCl; ▲, propadrine HCl; □, butadrine HCl; △, pentadrine.

DISCUSSION

These results, along with other data not described here, show that it is not yet possible to predict biological activity merely by measuring the change a compound produces in the electromotive force of a "model cell." Possibly the development of a more ideal cell may make for better correlation. Or perhaps the interesting correlations that have thus far been observed, e.g., those shown in Table I, are receiving incomplete or even misleading interpretations. Further studies with homologous series, as employed here, may clarify these points. Our results show only an approximate parallelism between the change in E. M. F. and the relative toxicities of homologous series.

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A Study of the Microbiological Assay of Riboflavin*

By HAROLD W. WOODSON,† ERNST R. KIRCH, and OLAF BERGEIM

The values determined using a mixture of twenty amino acids compared favorably with those obtained using the customary media containing alkali-treated peptone or casein hydrolyzate. For the riboflavin assay a new test organism, *Lactobacillus delbrückii*, gave essentially the same growth responses as the standard organism, *Lactobacillus casei*.

KNOWLEDGE of the growth factor requirements of various lactic acid bacteria, especially *Lactobacillus casei* and *Lactobacillus arabinosus 17-5*, has led to the development of useful microbiological methods for the quantitative determination of most of the B vitamins.

Microbiological assays of riboflavin, using as the test organism, *Lactobacillus casei*, have been adopted in many laboratories using the original method of Snell and Strong (1) or the modified medium of Silber and Unna (2). Frequency of irregular standard curves and discordant results are some of the difficulties which have been observed with the Snell and Strong medium, which contains alkali treated-photolyzed peptone and lead-treated yeast supplement. This has led to studies on the improvement of media for this determination, with the object of obtaining greater regularity and sensitivity in the response of the microorganism to riboflavin. In all of the suggested media along with the known growth factors are one or more materials of variable composition, i.e., hydrolyzed casein, photolyzed peptone, and yeast supplement. Recent studies on the amino acid requirements of *Lactobacillus casei* (3) and *Lactobacillus delbrückii LD5* (4), and the use of these organisms in microbiological amino acid assays (5–7) have suggested the possibility of using a completely synthetic medium of known and constant composition for vitamin assays.

Tomlinson and Peterson (8), in their experiments with a synthetic medium for the determination of biotin, suggest that the same medium could be used for the assay of riboflavin, pantothenic acid, and nicotinic acid. However, because of the longer time required (four days) and the possibility of other substances present in natural materials affecting the results, further experiments would be needed before adopting a synthetic medium for such assays.

In the present work, we were interested in the development of an amino acid medium for use in the determination of riboflavin and the application of *Lactobacillus delbrückii* to this assay.

EXPERIMENTAL

Organisms.- The organisms used are *Lactobacillus casei* and *Lactobacillus delbrückii* LD5. Stab stock cultures of the organisms are carried in a medium of the following composition: 1% glucose, 1.5% agar, and 1% yeast extract. Stock cultures are transferred weekly and are stored in the refrigerator.

Basal Media. The following media were used in this investigation: Silber-Unna (2) modification of the original Snell and Strong medium (1), Landy and Dicken (9), and the amino acid medium similar to that described by Baumgarten, *et al.* (10). The composition of the amino acid medium is shown in Table I.

This medium is double strength, 5 ml. being used per tube, diluted to 10 ml. with the solution being assayed plus water. The amino acid medium of Baumgarten, *et al.* (10) was selected since their experiments showed that other proposed amino acid media (3–6) did not give maximal growth response with *L. casei*, whereas maximal growth response was obtained with their medium by the addition of thiamine, which acts as a stimulating factor for *L. casei*.

Assay Procedure. Inoculum tubes are prepared by direct transfer from stock culture to 10 ml. of the diluted basal medium containing 1 μg. of riboflavin. After incubation for twenty-four hours at 37°, the culture is centrifuged and the cells are resuspended in 10 ml. of sterile physiological sodium chloride. One drop of this suspension is used per tube for inoculation.

Assays were carried out in 16 × 150-mm. Pyrex bacteriological culture tubes. To establish the standard curve, duplicate tubes were set up containing the following concentrations of riboflavin: 0.00, 0.05, 0.10, 0.15, 0.2, 0.3, and 0.5 μg. of riboflavin.

* Received April 29, 1946, from the Department of Biological Chemistry, University of Illinois College of Medicine, and the Department of Chemistry, University of Illinois College of Pharmacy, Chicago, Illinois.
This paper is based on a thesis submitted by Harold W. Woodson to the Graduate School of the University of Illinois in partial fulfillment of the requirements for the degree of Master of Science.
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Figure 3 shows the results obtained with propadrine, butadrine, and pentadrine (C_6H_5—CHOH—CH—R, R = CH_3, C_2H_5, and n-C_3H_7, respectively)
|
NH_2
hydrochlorides, using guaiacol and potassium chloride solution. Considered from the point of toxicity, the curves are in proper order, since pentadrine is about three times more toxic than propadrine (8).

Figure 2 shows the results obtained with choline chloride, methylcholine chloride and "triethylcholine" chloride, $HOCH_2CH_2$—$N(C_2H_5)_3Cl$, with sodium benzoate as the solution and nitrobenzene as the oil. Again, the change in voltage gives no indication of physiological activity, but the compound showing the greatest change in voltage is the most toxic.

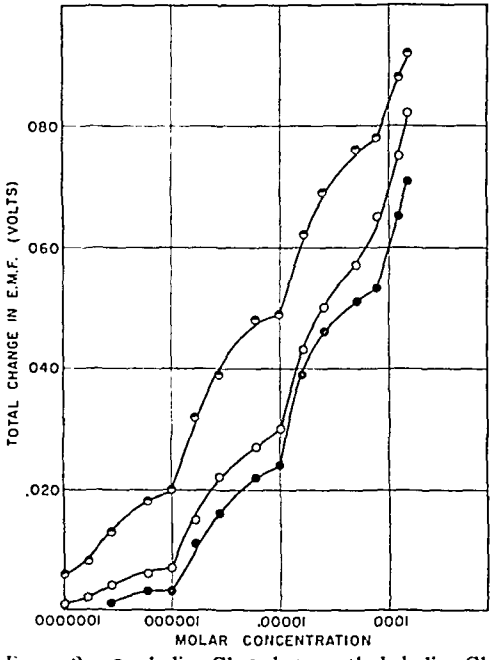

Figure 2.—●, choline Cl; ○, beta methyl choline Cl; ◓, triethyl choline Cl.

In Fig 3 are shown also the results obtained with cocaine hydrochloride and papaverine hydrochloride, the cell combination being guaiacol and potassium chloride. Here again, the more toxic cocaine produces the greater change in voltage.

DISCUSSION

These results, along with other data not described here, show that it is not yet possible to predict biological activity merely by measuring the change a compound produces in the electromotive force of a "model cell." Possibly the development of a more ideal cell may make for better correlation. Or perhaps the interesting correlations that have thus far been observed, e.g., those shown in Table I, are receiving incomplete or even misleading interpretations. Further studies with homologous series, as employed here, may clarify these points. Our results show only an approximate parallelism between the change in E. M. F. and the relative toxicities of homologous series.

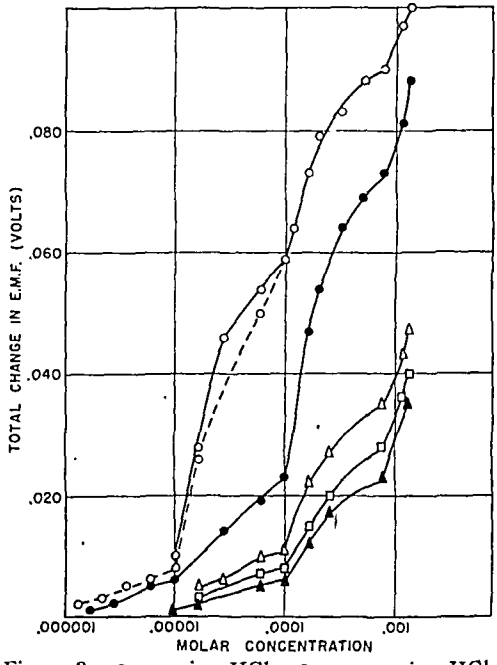

Figure 3.—○, cocaine HCl; ●, papaverine HCl; ▲, propadrine HCl; □, butadrine HCl; △, pentadrine.

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A Study of the Microbiological Assay of Riboflavin*

By HAROLD W. WOODSON,† ERNST R. KIRCH, and OLAF BERGEIM

The values determined using a mixture of twenty amino acids compared favorably with those obtained using the customary media containing alkali-treated peptone or casein hydrolyzate. For the riboflavin assay a new test organism, *Lactobacillus delbrückii,* gave essentially the same growth responses as the standard organism, *Lactobacillus casei.*

KNOWLEDGE of the growth factor requirements of various lactic acid bacteria, especially *Lactobacillus casei* and *Lactobacillus arabinosus 17-5,* has led to the development of useful microbiological methods for the quantitative determination of most of the B vitamins.

Microbiological assays of riboflavin, using as the test organism, *Lactobacillus casei,* have been adopted in many laboratories using the original method of Snell and Strong (1) or the modified medium of Silber and Unna (2). Frequency of irregular standard curves and discordant results are some of the difficulties which have been observed with the Snell and Strong medium, which contains alkali treated-photolyzed peptone and lead-treated yeast supplement. This has led to studies on the improvement of media for this determination, with the object of obtaining greater regularity and sensitivity in the response of the microorganism to riboflavin. In all of the suggested media along with the known growth factors are one or more materials of variable composition, i.e., hydrolyzed casein, photolyzed peptone, and yeast supplement. Recent studies on the amino acid requirements of *Lactobacillus casei* (3) and *Lactobacillus delbrückii LD5* (4), and the use of these organisms in microbiological amino acid assays (5–7) have suggested the possibility of using a completely synthetic medium of known and constant composition for vitamin assays.

Tomlinson and Peterson (8), in their experiments with a synthetic medium for the determination of biotin, suggest that the same medium could be used for the assay of riboflavin, pantothenic acid, and nicotinic acid. However, because of the longer time required (four days) and the possibility of other substances present in natural materials affecting the results, further experiments would be needed before adopting a synthetic medium for such assays.

In the present work, we were interested in the development of an amino acid medium for use in the determination of riboflavin and the application of *Lactobacillus delbrückii* to this assay.

EXPERIMENTAL

Organisms.- The organisms used are *Lactobacillus casei* and *Lactobacillus delbrückii* LD5. Stab stock cultures of the organisms are carried in a medium of the following composition: 1% glucose, 1.5% agar, and 1% yeast extract. Stock cultures are transferred weekly and are stored in the refrigerator.

Basal Media. The following media were used in this investigation: Silber-Unna (2) modification of the original Snell and Strong medium (1), Landy and Dicken (9), and the amino acid medium similar to that described by Baumgarten, *et al.* (10). The composition of the amino acid medium is shown in Table I.

This medium is double strength, 5 ml. being used per tube, diluted to 10 ml. with the solution being assayed plus water. The amino acid medium of Baumgarten, *et al.* (10) was selected since their experiments showed that other proposed amino acid media (3–6) did not give maximal growth response with *L. casei,* whereas maximal growth response was obtained with their medium by the addition of thiamine, which acts as a stimulating factor for *L. casei.*

Assay Procedure. Inoculum tubes are prepared by direct transfer from stock culture to 10 ml. of the diluted basal medium containing 1 μg. of riboflavin. After incubation for twenty-four hours at 37°, the culture is centrifuged and the cells are resuspended in 10 ml. of sterile physiological sodium chloride. One drop of this suspension is used per tube for inoculation.

Assays were carried out in 16 × 150-mm. Pyrex bacteriological culture tubes. To establish the standard curve, duplicate tubes were set up containing the following concentrations of riboflavin: 0.00, 0.05, 0.10, 0.15, 0.2, 0.3, and 0.5 μg. of riboflavin.

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Various amounts of the materials to be assayed, estimated to contain between 0.025 to 0.2 μg. of riboflavin, are pipetted into additional tubes. Volumes up to 5 ml. of the unknown material may be used. Unknown samples were run in duplicate at three levels. Unless otherwise noted the tubes were incubated at 37° for seventy-two hours and the amount of lactic acid produced was determined by titration with 0.1 *N* NaOH, bromothymol blue being used as the indicator.

Effect of Increasing Glucose and Buffer Content of Medium.—Following the suggestion of Stokes and Martin (11) the glucose content of the amino acid medium was increased from 1% to 2%, the sodium acetate from 0.6% to 1.8%. Contrary to their findings, there was no increase in acid production; in fact the curves obtained were very erratic.

Recovery Experiments.—Yeast extract (Difco) and a commercial B-complex elixir were assayed for riboflavin content using *L. casei* and *L. delbrückii* and the amino acid medium. Recoveries of riboflavin added to these materials are quantitative within the experimental error of microbiological methods (Table III).

TABLE I.—AMINO ACID BASAL MEDIUM

dl-Alanine	400 mg.	Glucose	20	Gm.
l (+)-Arginine	400 mg.	Sodium acetate (anhydrous)	12	Gm.
dl-Aspartic acid	400 mg.	Adenine	100	mg.
l (−)-Cystine	400 mg.	Guanine	100	mg.
l (+)-Glutamic acid	400 mg.	Uracil	100	mg.
Glycine	400 mg.	Inorganic salt solution A	10	ml.
l (−)-Histidine	400 mg.	Inorganic salt solution B	10	ml.
l (−)-Hydroxyproline	400 mg.	Thiamine	2	mg.
dl-Isoleucine	400 mg.	Pyridoxine	0.4	mg.
l(−)-Leucine	400 mg.	Calcium pantothenate	0.4	mg.
dl-Lysine	400 mg.	Nicotinic acid	0.8	mg.
dl-Methionine	400 mg.	*p*-Aminobenzoic acid	0.4	mg.
dl-Norleucine	400 mg.	Biotin	10	μg.
dl-Phenylalanine	400 mg.	Folic acid[a]	25	μg.
l (−)-Proline	400 mg.	Adjust to *p*H 6.6–6.8		
dl-Serine	400 mg.	Add distilled H_2O to	1000	ml.
dl-Threonine	400 mg.			
l (−)-Tryptophane	400 mg.			
l (−)-Tyrosine	400 mg.			
dl-Valine	400 mg.			

[a] Equivalent to 25 μg. of material of "potency 3100." Kindly supplied by Dr. R. J. Williams of the University of Texas.

Lactobacillus delbrückii as a Test Organism.—Although *Lactobacillus casei* is the standard organism used in the riboflavin assay, it seemed desirable to investigate the possibility of using another lactic acid bacteria. Stokes and Gunness (4, 7) have described the amino acid requirements of *L. delbrückii LD5*, and have used the organism for the assay of aspartic acid and serine. Their work has also confirmed the fact that riboflavin is essential for *L. delbrückii*. Therefore, the growth response of *L. delbrückii* to riboflavin in comparison with *L. casei* on Silber-Unna medium and the amino acid medium was studied.

Comparison of Basal Media.—Growth response of *L. casei* and *L. delbrückii* on the three basal media, previously described, were studied as a controlled experiment. Assay tubes were set up in duplicate and all operations, solutions, and inocula were the same. The results of this experiment, as shown in Table II, indicate that there is fairly close correlation of growth response in the media tested.

TABLE II.—COMPARISON OF ACID PRODUCTION WITH DIFFERENT SOURCES OF NITROGEN. ml. 0.1 *N* ACID PER 10 ml. OF CULTURE MEDIUM

γ per ml. Riboflavin	Silber-Unna	Amino Acid	Landy-Dicken
	Lactobacillus casei		
0.00	1.2	1.55	1.4
0.05	3.9	4.85	4.7
0.1	5.5	6.15	6.2
0.15	6.9	6.8	6.6
0.2	8.4	7.6	8.2
0.3	9.5	8.55	9.0
0.5	10.3	9.1	10.5
	Lactobacillus delbrückii		
0.00	1.1	1.55	1.4
0.05	3.6	4.70	4.8
0.1	5.4	5.9	5.8
0.15	6.9	6.7	6.6
0.2	8.2	7.5	7.6
0.3	9.6	9.2	9.0
0.5	9.9	9.6	10.0

Effect of Time on Acid Production.—Since it was previously reported by Tomlinson and Peterson (8) that a four-day incubation period was necessary for satisfactory growth on their synthetic medium for the assay of biotin, experiments were conducted with *L. casei* on the amino acid medium. With an incubation period of ninety-seven hours, practically the same results were obtained as those following the seventy-two hour incubation period.

Comparative Riboflavin Assays.—In order to have a basis for evaluating the amino acid medium, a number of materials of widely different composition have been assayed in triplicate with this medium, and also that of the Silber-Unna modification (2) of the original Snell and Strong medium (1). In all cases, the

TABLE III.—RECOVERY OF RIBOFLAVIN

Material	Riboflavin Content, γ per Tube	Riboflavin Added, γ per Tube	Total, γ per Tube	Riboflavin Recovered, γ per Tube	Recovery, %
		Amino Acid Medium			
		Lactobacillus casei			
Yeast extract	0.06	0.2	0.26	0.255	98
B-Complex elixir	0.11	0.1	0.21	0.205	98
		Lactobacillus delbrückii			
Yeast extract	0.07	0.1	0.17	0.17	100
B-Complex elixir	0.11	0.1	0.21	0.19	90

values for riboflavin content were in essential agreement with the results obtained by the Silber-Unna medium (Table IV). The assays of the milk, urine, and yeast extract were carried out on the untreated material diluted to the proper concentration.

TABLE IV.—COMPARATIVE RIBOFLAVIN ASSAYS ON VARIOUS MATERIALS

Material	Silber-Unna Medium, *L. casei*, *μg. per Gm. or Ml.*	Amino Acid Medium *L. casei*, *μg. per Gm. or Ml.*
Milk	2.06	2.31
Urine	1.50	1.50
Yeast extract	55.00	60.00

SUMMARY

1. An amino acid medium is described for use in the microbiological assay of riboflavin. The alkali-treated peptone or casein hydrolyzate of the usual media is replaced by a mixture of 20 amino acids at the level of 2 mg. per tube of 10 ml. There is no improvement in the medium by increasing the glucose or buffer capacity.

2. A new test organism for the riboflavin assay, *Lactobacillus delbrückii*, is described which gives comparable growth responses with the standard organism, *Lactobacillus casei*.

3. Various materials, assayed with the amino acid medium, gave values which compared favorably with those obtained by a standard medium.

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Book Reviews

American Pharmacy. Edited by R. A. LYMAN. J. B. Lippincott Co., Philadelphia, 1945. xix + 545 pp. 18 × 26 cm. 197 illus. Price, $8.00.

American Pharmacy is the result of the efforts of twenty-eight men in pharmacy to produce a modern textbook for beginning students in pharmacy. The book is divided into three parts comprising thirty chapters and covering 525 pages of text. In producing this work the following points were considered:

The book was to be (1) primarily a teaching tool and not a book of encyclopedic nature, (2) a reference work for the practicing pharmacist and others in the medical field, (3) modern in every respect, with obsolete material being deleted, and (4) concise and not a reproduction of the official books.

In selecting the various authors an at-

tempt was made to secure men who were specialists in a particular phase of pharmacy and who were cognizant of the needs of pharmacy as a whole.

Since this book is intended primarily as a teaching aid, the comments of the review are so directed.

Part one of the book deals with the Fundamental Principles and Processes of Pharmacy. These are Metrology; Specific Gravity and Specific Volume; Heat and Refrigeration; Purification and Clarification; The Mechanical Subdivision of Drugs; Solutions; Colloids, Emulsions and Suspensions; Extractions; Bacteriologic Technique; and Preservation and Packaging. Of these chapters, Heat and Refrigeration; Solutions; Extractions; and Mechanical Subdivision of Drugs are good for freshman study. Metrology and Specific Gravity and Specific Volume are a little brief. They do not include a sufficient number of illustrations to aid the student in mastering these subjects. The chapters on Purification and Clarification; and Colloids, Emulsions and Suspensions are excellent, but they are probably too advanced for beginning students. There seems to be little or no justification for the chapter on Bacteriologic Technique since this subject is taught in a separate course and later than the freshman year in most colleges.

Part two of the book deals with Pharmaceutical Preparations. The material is grouped under the conventional headings, such as Waters, Syrups and Juices; Solutions; Injections; Infusions and Decoctions; Mucilages, Creams, Glycerogelatins, Glycerites and Collodion; Mixtures, Magmas and Gels; Soaps and Oleates; Liniments, Lotions, Petroxolins, and Sprays; Ointments, Cerates, Plasters, and Cataplasms; Emulsions; Suppositories; Spirits and Elixirs; Vinegars, Tinctures, Fluidextracts, Fluidglycerates and Extracts; Resins and Oleoresins; Masses, Pills, Troches and Tablets; Powders, Capsules, Cachets, Oil Sugars, Candy Medication and Triturations; and Effervescent Salts. In general, each of these subjects is treated well. There are some differences among the authors in the amount of emphasis placed on the techniques involved in the preparation of these pharmaceuticals and some authors seem to stress the use of the preparation more than the manufacture of it. Here again it is noted that there is a wide difference in the levels of discussion of each group of preparations. For example, the chapter on Ointments, Cerates, Plasters and Cataplasms is excellent but hardly within the grasp of a student who is just starting his pharmaceutical studies. The chapter on Soaps and Oleates is of a similar nature. The order of arrangement of the chapters is purely arbitrary and they do not seem to have any particular connection with each other. Not all official items are discussed; this is particularly true of the Chapter on Tinctures, Fluidextracts, etc.

Part three of the book contains chapters on Vitamins; Hormones and Endocrine Glands; and Introduction to Biologicals. These chapters are considered all too brief to be of value in pharmaceutical education, particularly in those colleges that give two or more credit hours in each of these areas. Here again, this material is better suited to advanced students.

However, the book should serve as a valuable reference to students, pharmacists, and others interested in the medical sciences.—E. P. GUTH.

Howell's Textbook of Physiology, 15th Edition. Edited by J. F. FULTON, with ten collaborators. W. B. Saunders Co., Philadelphia, 1946. xxxv + 1304 pp. 15 x 23 cm. Price, $8.00.

When a textbook reaches its fifteenth edition, it would scarcely seem necessary for it to be reviewed. However, *Howell's Textbook of Physiology*, with this edition, has been rewritten completely by ten collaborators under the able editorship of Dr. J. F. Fulton.

The text covers the entire field of physiology, and the sections are developed in the usual pattern. Such an extensive book cannot be reviewed in detail with satisfaction; it is rather to be read and used. In short, the book is thoroughly modernized, and although written by many authors, its continuity is excellent.—MELVIN W. GREEN.

Scientific Edition

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A Study of Penicillin Ointments and Creams*

By M. L. NEUROTH,† C. O. LEE, JOHN E. CHRISTIAN, and GLENN L. JENKINS

A number of ointments and cream formulas containing penicillin were prepared and studied for their antibactericidal activity against organisms. Tests were made by a new modified agar cup-plate method which was devised in the laboratories of the School of Pharmacy at Purdue University.

INTRODUCTION

IN A PAPER dealing with the topical forms for the application of penicillin Lesser (1) stated that the greatest use of it by the general practitioner would be in the form of ointments and creams. Much smaller concentrations of penicillin are needed for local applications than are required for parenteral use.

The advantages of the topical application are several, such as (*a*) economy, (*b*) greater concentration at the site of infection, (*c*) the effect of a single dose may be greatly prolonged, (*d*) the response to treatment is more prompt, and (*e*) the drug is held in contact with the tissues longer when in the form of an ointment than when in aqueous solution. Lesser stated further that when the two basic problems, namely, the most suitable base and a more stable form of penicillin, were solved greater use of penicillin locally would be made.

In the preparation of a penicillin ointment Burns (2) stated that the prime objective was to insure therapeutic activity and that the base used should not only be smooth but as nearly neutral as possible. Such a preparation should also withstand sterilization without much separation and re-emulsify easily upon cooling. Penicillin ointments should be kept as cool as possible.

Greey and Hebb (3) studied nine different penicillin ointment formulas. The most stable formula was composed of stearic acid, fatty acid esters of sorbitan, and about 77.5 per cent of water.

Mentis (4) obtained excellent results in the treatment of impetigo on infants with a penicillin ointment composed of 500 units per gram of penicillin in Abbott's hydrosorb base. On the other hand, Templeton (5)

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and others tested several ointments of penicillin, by the agar cup-plate method, using aquaphor as the base, and little or no antibacterial activity was indicated. Reports such as these would make it seem that the character of the base makes a difference in the activity of penicillin when used topically.

Payne (6) used a penicillin ointment, composed of 200 units per gram in a base of Lanette Wax 5x with 30 per cent water. He observed that if this was prepared in a copper pan it lost all of its antibacterial activity within three days but if prepared in a glass container the activity was more prolonged.

Dahl (7) reported a number of bases which were satisfactory for use with penicillin. Differences in the retention of sodium penicillin by various bases was noted. Calcium penicillin was preferable to sodium penicillin for ointments. The potency of penicillin ointments was longest in anhydrous bases.

The Massachusetts College of Pharmacy (8) studied the problem of ointment bases for penicillin. Bases of the oil-in-water type were found to be the best vehicles especially when the water content was as much as 70 per cent.

The statement has been made that for the treatment of burns and other superficial wounds, as well as for various skin infections, it is desirable to apply penicillin in a semisolid base. A base composed of equal parts of Lanette wax, soft petrolatum, and water has been proposed as being suitable (9). Lanette wax is a British product not readily available here but substitutes for it have been proposed.

EXPERIMENTAL

The requirements of a satisfactory base for the topical application of penicillin have been reviewed. The object of this research has been to study various ointment and cream bases with penicillin incorporated in them in an effort to develop a formula in which the activity of penicillin could be maintained over an extended period of time under refrigeration.

In the course of this study a modification of the agar cup-plate method was developed (10). The new method is simple and unique. It makes use of a heating element in the formation, in a sterile manner, of uniform agar cups. This is accomplished by standing flat-end Pyrex glass rods, 10 mm. in length and any desired diameter, in liquid agar. The agar is allowed to harden and each of the glass rods is then heated by means of a small electric heating element which, in the form of a loop, is slipped down over the exposed upper end of the rod. The rod, upon becoming warm, melts a surrounding film of the agar in which it stands, and is removed. The agar which melted at the base of the rod forms an agar seal at the bottom of the cup.

Four such cups were made in each Petri dish. Into one cup was placed about 0.50 Gm. of the control composed usually of the unmedicated base. Ointments in 0.50-Gm. amounts and in varying concentrations of penicillin were placed in the other three cups. Next 5 cc. of inoculated agar was poured over the top of the base layer and allowed to cool. The inoculated agar consisted of 2 cc. of a sixteen- to eighteen-hour beef broth culture of Staphylococcus aureus in 100 cc. of melted agar. The Petri dish was then placed in an incubation oven in an inverted position and kept at 37° for sixteen to eighteen hours.

The clear zones around the cups were very uniform and varied in diameter according to the concentration of the penicillin in the ointment. Figure 1 shows the heating unit being used to remove one of the glass rods. Figure 2 shows the seeded agar plate after being incubated. The clear zones are an indication of the antibacterial activity of the penicillin ointment.

FIGURE 1.

Preparation and Testing of Penicillin Ointments.—In the course of this study many ointment formulas were prepared and tested. Only three of them are reported in this paper. The formulas for these bases will be given, following which will be

tables summarizing the antibacterial activity of the ointments as indicated by a modified agar cup-plate method. Inasmuch as these formulas are pharmaceutical preparations which should be compounded *secundum artem*, the procedures for preparing them will be omitted. The data given in the tables are largely self-explanatory.

Sodium penicillin was dissolved in a minimum amount of water and then incorporated into the bases. When those ointments which contained the penicillin in therapeutic concentrations were tested the plates showed no growth of the organisms.

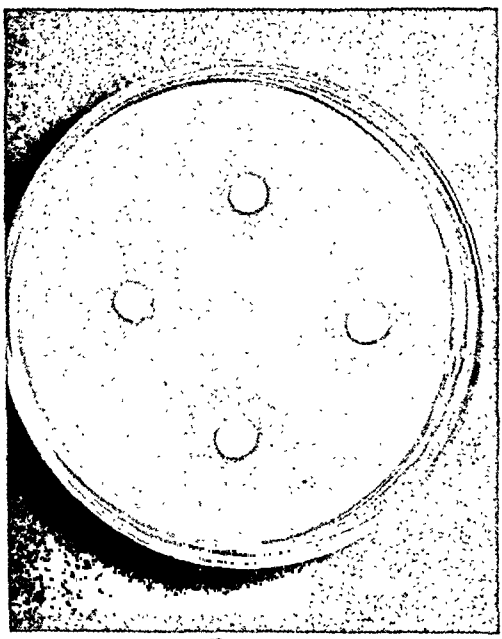

FIGURE 2.

Therefore it was necessary to make tests upon ointments with a very low concentration of penicillin. The concentrations used were as follows: (*a*) one unit per gram, (*b*) one-half unit per gram, (*c*) one-fourth unit per gram, (*d*) control—the base. Portions of about 30 Gm. of each of the above ointments were made and tested at the time prepared and at intervals of several days. This was to observe the loss of antibacterial potency with aging. The ointments were refrigerated during the test period.

FORMULA 1

Cetyl alcohol	4.50 Gm.
Sodium lauryl sulfate	0.50 Gm.
Stearyl alcohol	12.90 Gm.
White petrolatum	17.40 Gm.
Cetaceum	17.40 Gm.
Tween 20	2.30 Gm.
Sterile distilled water	45.00 Gm.

This base was used to prepare the three dilute ointments of penicillin as described above. Their antibacterial activity upon an eighteen to twenty-four hour culture of S. aureus, 209 P. is indicated in Table I. The plain base was used as the control except when otherwise indicated by the agar cup-plate method.

TABLE I

Dates	Ointments with Varying Concentrations of Penicillin, Units/Gm.	Antibacterial Activity Indicated by Clear Zones Measured in Mm. Plates 1	2	3	4
9-22-45	Control[a]	42	43	44	44
9-27-45	Control	0	0	0	0
10-3-45	Control	0	0	0	0
9-22-45	1	26.5	26	27.5	28
9-27-45	1	22	22.2	22	22
10-3-45	1	16	16	15	16
9-22-45	1/2	22	21.5	22	21.5
9-27-45	1/2	13	..	13	11
9-22-45	1/4	20	21	19	21
10-3-45	1/4	12.25	10.5	11	11

[a] The control in this instance was an ointment composed of 250 units of penicillin per gram and illustrates the fact that it is not practicable to test ointments of high penicillin concentration.

Comments.—Two observations may be made from the results indicated in Table I. The first is that the width of the clear zones is in direct proportion to the concentration of penicillin in the ointment. The second is that the activity of the penicillin in the ointment decreased with age even though stored at a low temperature.

FORMULA 2

Stearic acid	20.0 Gm.
Span 60	14.4 Gm.
Span 80	2 0 Gm.
Tween 20	5.6 Gm.
Sterile distilled water	138.0 Gm.

This formula resulted in a soft cream and was suggested by Greey and Hebb (3). The activity of penicillin in this base was tested after the manner described above. The results are given in Table II.

TABLE II

Dates	Creams with Varying Concentrations of Penicillin, Units/Gm.	Antibacterial Activity Indicated by Clear Zones Measured in Mm. Plates 1	2	3	4
8-24-45	Control	0	0	0	0
9-5-45	Control	0	0	0	0
9-8-45	Control	0	0	0	0
9-22-45	Control (250 units)	37	36	37	36
8-24-45	1	26.5	27.5	24.0	23.5
9-5-45	1	26.0	26.0	25.5	24.0
9-8-45	1	26.5	27.5	24.0	23.5
9-22-45	1	17.0	17.0	16.5	18.5
8-24-45	1/2	23.5	24.5	22.0	21.0
9-5-45	1/2	23.5	24.0	24.0	24.0
9-8-45	1/2	23.5	24.5	22.0	21.0
9-22-45	1/2	16.0	16.5	16.0	15.5
8-24-45	1/4	20.0	20.0	18.0	17.0
9-5-45	1/4	22.5	24.0	22.0	24.0
9-8-45	1/4	20.0	20.0	18.0	17.0
9-22-45	1/4	10.0	9	10.0	9.0

Comments.—It should be observed that the control which consisted of the base only gave no evidence of any antibacterial action. The control to which 250 units of penicillin was added showed marked activity. The creams with the 1/4 unit of penicillin per gram showed less antibacterial activity than the stronger creams. All creams maintained activity for about sixteen days. By the thirtieth day all had shown a decided loss in bacterial activity. The ointments were stored at refrigerator temperature while being studied.

FORMULA 3

Cetyl alcohol	21.0 Gm.
Cetaceum	12.1 Gm.
White petrolatum	21.0 Gm.
Sodium lauryl sulfate	0.3 Gm.
Sodium citrate	3.0 Gm.
Urea	4.9 Gm.
Sterile distilled water	37.7 Gm.

This proved to be a satisfactory base. It was used to prepare penicillin ointments of the same concentrations as those reported above, which were stored at refrigerator temperature while being tested.

The antibacterial activity of the ointments prepared from this base are given in Table III.

TABLE III

Dates	Ointments with Varying Concentrations of Penicillin, Units/Gm.	Antibacterial Activity Indicated by Clear Zones Measured in Mm. Plates 1	2	3	4
9-14-45	Control	0	0	0	0
9-27-45	Control	0	0	0	0
9-14-45	1	19.50	19.75	19.50	20.50
9-27-45	1	20.00	20.50	20.25	19.75
9-14-45	1/2	16.50	16.50	17.90	16.50
9-27-45	1/2	16.50	16.00	15.50	16.25
9-14-45	1/4	13.00	13.50	13.00	12.50
9-27-45	1/4	12.50	12.00	12.75	14.00

Comments.—These ointments were tested but twice, thirteen days apart, and showed very little deterioration as to antibacterial activity during that time. A more prolonged study of the activity and stability of penicillin in this base should be made.

Other Formulas.—In addition to these three formulas we studied, in the same manner, the antibacterial activity of penicillin in (*a*) Simple ointment U. S. P. XI, (*b*) Hydrosorb, (*c*) Aquaphor, and (*d*) Aquaphor with 50% water incorporated. The test results were all negative indicating that these bases were not satisfactory for penicillin in low concentrations.

The penicillin was extracted from the Aquaphor-water ointment and tested. The results indicated that the penicillin had not been destroyed in the ointment but was rendered inactive in the base in the concentrations used.

SUMMARY AND CONCLUSIONS

Many ointments and creams containing penicillin in concentrations of one unit, one-half unit, and one-fourth unit per gram have been tested. The most satisfactory formulas were those of the oil-in-water type emulsions containing some of the newer wetting agents. The presence of water in concentrations as high as 70 per cent in these bases, if kept at refrigerator temperatures, was not detrimental to penicillin. In fact those of high water content were the most satisfactory.

The new modified technique which we have proposed for the agar cup-plate test makes it possible to evaluate the activity of penicillin without first having to extract it from the ointment. The availability of penicillin in ointments may be determined by observing the zones of inhibition on the agar plates.

The penicillin was protected in the ointments by the addition of small amounts of sodium citrate and urea. It was fouud that the ointment could be sterilized by the method offered by Payne (6).

This study has been made upon ointments and creams with very low concentrations of penicillin, which deteriorated rather rapidly at room temperature. However, ointments with high or therapeutic concentrations retained their antibacterial activity even after considerable exposure to room temperature.

Finally we believe that this study indicates that a method of assay for penicillin in ointments and creams, by dilution after the manner of testing solutions, is possible. This would necessitate the development of a standard base.

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A Simple Pipette Washer for the Average Laboratory*

By JOHN H. BREWER

MANY DEVICES have been suggested for washing pipettes and there are several available on the open market. For the most part they have too great capacity and are too expensive for the average laboratory. Most of them operate as an automatic fill and drain siphon. The design herein described works on a similar principle but is unique in that it is one piece and may be used with any 1000-cc. graduate or cylinder which has a depth of about 16 inches. It differs from other designs in that it is not necessary to have a hole in the cylinder or container below the top rim or to have the water in the vessel rise above the siphon tube in order to start the siphon. This pipette washer also differs from the usual design, such as that described by Wadsworth (1) in that the water enters the cylinder through the same tube through which it is later discharged.

The siphon (Fig. 1) is very easily constructed of glass in the laboratory, although one made of metal or plastic has been found to give better service in that it is not readily broken. Tubing with a $^5/_{16}$-inch inside diameter has been found satisfactory and may be bent as shown. In use a piece of glass cloth or wire gauze should be placed in the bottom of the cylinder, or graduate, to prevent breaking of the pipette tips and to insure proper washing.

Connect rubber tubing to siphon at point *A* (see diagram) and to water supply. Place tube *B* inside of cylinder containing pipettes to be washed and adjust water flow so that a liter graduate fills in approximately five minutes. The pipette washer will then operate flushing and refilling continuously. The washing operation should be conducted in a laboratory sink or on a drainboard.

Irregularities in water pressure may cause the cylinder to overflow. If this occurs and the water pressure cannot be controlled, it is recommended that a pressure regulating valve, set for one pound, be placed in the line.

Should it be necessary to wash a larger number of pipettes than can be held in a liter graduate, a Pyrex cylinder, six inches in diameter, is available and a stainless steel rack has been constructed to facilitate handling of the pipettes.

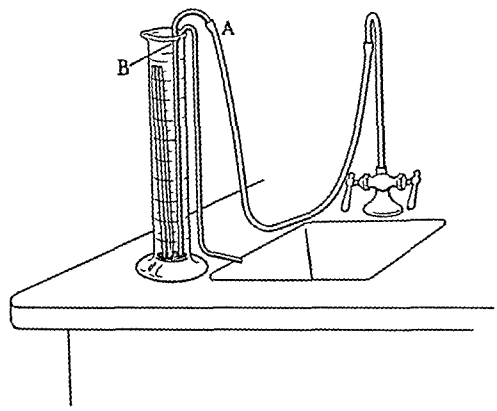

Fig. 1.—Pipette Washer

A good technique in an average size laboratory is to place the wire or glass gauze in the bottom of the cylinder and fill it half-full of disinfectant solution. This will prevent blood, milk, and other solutions from drying on the pipettes and making them hard to clean. At the close of the day the cylinder is carried to the sink and the pipette washer inserted and turned on. The next morning the pipettes will have rinsed about one hundred to two hundred times. To wash milk pipettes or other hard to clean or greasy ones, Calgonite with a wetting agent, or other good cleaning powders, should be used instead of lysol or other disinfectant solutions.

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* Received Aug. 15, 1946, from the Biological Research Laboratory, Hynson, Westcott & Dunning, Inc., Baltimore, Md.

The Synthesis of Chemotherapeutic Agents. I.* The Synthesis of Certain Thio and Dithio Compounds†,‡

By JOHN E. CHRISTIAN, GLENN L. JENKINS, LeROY C. KEAGLE, and JEAN ANN CRUM

Investigation of 5,5′-diacetamido-8,8′-diquinolyl disulfide indicated that it possesses a relatively high antimalarial activity. The report of this activity stimulated interest in the possible chemotherapeutic value of certain related thio and dithio compounds. This paper, the first of a series, describes the synthesis of sixteen compounds, eight of which are new, related to 5,5′-diacetamido-8,8′-diquinolyl disulfide. Twelve of these compounds were tested for antimalarial activity and the results of these tests are tabulated. In addition some of the compounds were also subjected to screening tests against Trypanosoma brucei, influenza virus and tetanus toxin.

A REPORT on the relatively high antimalarial activity of 5,5′-diacetamido-8,8′-diquinolyl disulfide (1) aroused considerable interest in the synthesis and chemotherapeutic activity of certain related thio and dithio compounds. This paper is the first of a series describing the synthesis and chemotherapeutic activity of a number of these compounds.

The compounds reported here have been prepared in an effort to obtain substances of higher intrinsic activity and with better pharmacological properties. In addition to the usual antimalarial tests, some of these compounds have been subjected to screening tests against Trypanosoma brucei, influenza virus, and tetanus toxin.

Most of the compounds have been synthesized by methods described by Christian and Jenkins (3, 4). Compounds containing active halogens were treated with sodium disulfide, splitting out sodium chloride to form thio or dithio compounds. If there were nitro groups present, these were reduced to the corresponding amino compounds using stannous chloride and hydrochloric acid or sodium sulfide.

EXPERIMENTAL

1. **5,5′-Diamino-8,8′-diquinolyl Disulfide.**—Two reduction procedures were found to give more satisfactory yields than previously reported. (A) A modification of the procedure using stannous chloride and hydrochloric acid described by Christian and Jenkins (2) and (B) the use of sodium sulfide (3).

Procedure A.—Three-tenths gram of 5,5′-dinitro-8,8′-diquinolyl disulfide, the preparation of which was reported in a previous reference (4), was added with stirring all at once to a solution of 11.5 Gm. of $SnCl_2.2H_2O$ in 12 cc. of concentrated HCl cooled to 5°. The mixture was heated on a steam bath for one hour, cooled, and placed in the cold overnight. The precipitate was collected, suspended in 20 cc. of water, and 27 cc. of 50% NaOH added with cooling. Water (140 cc.) was added to insure complete solution of the sodium stannate formed. The solution was filtered and air bubbled slowly through the filtrate for twenty-four hours (hydrogen peroxide may be used instead of air for the oxidation of the thiol to the disulfide). The yellow disulfide which precipitates was filtered and recrystallized from acetone and water. The pure crystals melt at 225–226° with decomposition.

2. **5,5′-Diacetamido-8,8′-diquinolyl Disulfide.**—The procedure used was that described by Christian and Jenkins (2).

3. **5,5′-Bis(*p*-acetamido-benzenesulfonamido)-8,8′-diquinolyl Disulfide.**—The procedure used was that described by Christian and Jenkins (2).

4. **6,6′-Dinitro-2,2′-diquinolyl Sulfide.**—The starting material, 2-chloro-6-nitroquinoline, for this reaction was supplied by the Committee on Medical Research (CMR) of the Office of Scientific Research and Development (OSRD). On recrystallization from alcohol and acetone this substance melted at 235° (total immersion).

To 1000 cc. of ethyl alcohol was added 20 Gm. of 2-chloro-6-nitroquinoline and the mixture heated to reflux. Through a dropping funnel placed above the condenser a hot alcoholic solution of Na_2S_2, prepared by dissolving 11.2 Gm. of crystalline sodium sulfide ($Na_2S.9H_2O$) and 1.5 Gm. of sulfur in 115 cc. of hot alcohol, was added over a period of

* For a previous paper in this series, see Christian and Jenkins, THIS JOURNAL, 34, 147–49(1945).

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‡ The work described in this paper was done in collaboration with Eli Lilly and Company and the Committee on Medical Research of the Office of Scientific Research and Development.

one hour to the boiling mixture. After the addition was complete, the alcoholic mixture was refluxed for four hours. The mixture was cooled and the product separated by filtration, washed with water, and dried; 18.5 Gm. yield (48% of the theoretical). Several recrystallizations from nitrobenzene or a mixture of nitrobenzene and pyridine yielded lemon-yellow crystals melting at 254–255° (total immersion).

Anal.—Calcd. for $C_{18}H_{10}N_4O_4S$: N, 14.82%. Found: N, 14.22%.

5. **6,6′-Diamino-2,2′-diquinolyl Sulfide.**—To a solution of 46 Gm. of $SnCl_2.2H_2O$ in 48 cc. of concentrated HCl cooled to 5° was added all at once with stirring 4.3 Gm. of 6,6′-dinitro-2,2′-diquinolyl sulfide. The mixture was heated on a steam bath for two hours, cooled, and filtered. The tin salt collected was suspended in 80 cc. of water, and 110 cc. of 50% NaOH added with cooling. Water (500 cc.) was added and the mixture filtered. The yellow precipitate was recrystallized from acetone and water using decolorizing charcoal to aid purification. Yield 5.5 Gm. (53.7% of the theoretical), m. p. 241–242° (total immersion).

Anal.—Calcd. for $C_{18}H_{14}N_4S$: S, 10.06%. Found: S, 10.10%.

6. **8,8′-Dinitro-7,7′-diquinolyl Sulfide.**—The starting material, 7-chloro-8-nitroquinoline, was supplied by CMR; m. p. 182–184°.

To 200 cc. of alcohol was added 20 Gm. of 7-chloro-8-nitroquinoline and the mixture heated to reflux. A hot alcoholic solution of Na_2S_2, prepared by dissolving 11.1 Gm. of $Na_2S.9H_2O$ and 1.5 Gm. sulfur in 110 cc. of hot alcohol, was added over a period of one hour. After the addition was complete the mixture was refluxed for four hours. The mixture was cooled and the product separated by filtration, washed with water and dried; 14.5 Gm. yield (38% of the theoretical). Several recrystallizations from pyridine and alcohol yielded a product melting at 265–266° (total immersion).

Anal.—Calcd. for $C_{18}H_{10}N_4O_4S$: S, 8.47%; N, 14.82%. Found: S, 8.63%; N, 14.09%.

7. **8,8′-Diamino-7,7′-diquinolyl Sulfide.**—Pure 8,8′-dinitro-7,7′-diquinolyl sulfide (13.3 Gm.) was added slowly with cooling to a solution of 72 Gm. $SnCl_2.2H_2O$ in 70 cc. of concentrated HCl. The mixture was heated on a steam bath for two hours, cooled, filtered, and dried. Twenty-nine grams of the tin complex was obtained. This complex was heated with 250 cc. of 2.0% NaOH and the mixture extracted with acetone. The product was precipitated from the acetone upon the addition of water; 9.4 Gm. crude sulfide. Several recrystallizations from acetone and water mixtures yielded 6.5 Gm. (56.7% of the theoretical) of pure sulfide melting at 149–50° (total immersion).

Anal.—Calcd. for $C_{18}H_{14}N_4S$: S, 10.06%; N, 17.61%. Found: S, 9.80%; N, 17.49%.

8. **β,β′-Dianthraquinone Disulfide.**—β-Chloroanthraquinone (30 Gm.) was dissolved in 150 cc. of refluxing alcohol and to this hot solution was added a warm solution of Na_2S_2, prepared by dissolving 14.6 Gm. $Na_2S.9H_2O$ and 1.9 Gm. sulfur in 140 cc. of alcohol. The mixture was refluxed for four hours, cooled, and filtered. The precipitate was washed with water and alcohol and dried. The product, 2.3 Gm. (39% of theory), was recrystallized from a pyridine and alcohol mixture, m. p. 264–265° (total immersion). Gatterman (10) reported a melting point of 257°.

Anal.—Calcd. for $C_{28}H_{14}O_4S_2$: S, 13.39%. Found: S, 13.34%.

9. **2,2′-Dichloro-dibenzyl Disulfide.**—This compound was prepared by Speroni (5) by a different procedure. *o*-Chlorobenzyl-chloride (30 Gm.) was dissolved in 150 cc. of refluxing alcohol. To the hot alcoholic solution a warm solution of Na_2S_2, prepared by dissolving 21.8 Gm. of $Na_2S.9H_2O$ and 2.9 Gm. sulfur in 210 cc. of hot alcohol, was added slowly. The refluxing was continued for one hour and the mixture allowed to cool. The precipitate was washed with water and recrystallized from alcohol; 28 Gm. (96% of theory), m. p. 89–90° (total immersion).

10. **4,4′-Dichloro-dibenzyl Disulfide.**—This compound was prepared by Jackson and White (6). *p*-Chlorobenzylchloride (30 Gm.) was dissolved in 150 cc. of refluxing alcohol, and the procedure carried out as described above. The product, 27 Gm. (93% of theory), was recrystallized from alcohol; m. p. 58–59° (total immersion).

11. **3,3′,4,4′-Tetrachloro-dibenzyl Disulfide.**—3,4-Dichlorobenzyl chloride (30 Gm.) was refluxed with 150 cc. of alcohol for one-half hour. To the hot alcoholic solution was added a warm solution of Na_2S_2, prepared by dissolving 17.9 Gm. of $Na_2S.9H_2O$ and 2.4 Gm. sulfur in 170 cc. of hot alcohol. The refluxing was continued for one hour and the mixture allowed to cool. The precipitate which formed was washed with water and recrystallized from alcohol; 26 Gm. (89% of theory), m. p. 94–95° (total immersion).

Anal.—Calcd. for $C_{14}H_{10}Cl_4S_2$: S, 16.66%; Cl, 37.00%. Found: S, 16.81%; Cl, 37.08%.

12. **2,2′,4,4′-Tetrachloro-dibenzyl Disulfide.**—2,4-Dichlorobenzyl chloride (30 Gm.) was refluxed with 150 cc. of alcohol for one-half hour, and the procedure continued as described above. The product, 25 Gm. (84% of theory), was recrystallized from alcohol; m. p. 74–75° (total immersion).

Anal.—Calcd. for $C_{14}H_{10}Cl_4S_2$: S, 16.66%; Cl, 37.00%. Found: S, 16.75%; Cl, 37.09%.

13. **8,8′-Dinitro-5,5′-diisoquinolyl Sulfide.**—Ethyl alcohol (200 cc.) and 20 Gm. of 5-chloro-8-nitro-isoquinoline were placed in a flask and refluxed for one-half hour. To this hot solution was added slowly a warm solution of Na_2S_2, prepared by dissolving 11.1 Gm. of $Na_2S.9H_2O$ and 1.5 Gm. of sulfur in 110 cc. of hot alcohol. The mixture was refluxed for four hours, cooled, and filtered. The yellow product, 15.8 Gm. (40.2% of theory), was recrystallized from pyridine and alcohol; m. p.

242–244° (total immersion). Attempts to reduce this compound to the corresponding amino compound failed.

Anal.—Calcd. for $C_{18}H_{10}N_4O_4S$: S, 8.47%; N, 14.82%. Found: S, 8.80%; N, 14.97%.

14. 5,5′-Dinitro-1,1′-diisoquinolyl Disulfide.—1-Chloro-5-nitroisoquinoline (20 Gm.) was dissolved in 500 cc. of hot alcohol. To this hot solution was added a warm solution of Na_2S_2, prepared by dissolving 11.1 Gm. $Na_2S.9H_2O$ and 1.5 Gm. sulfur in 110 cc. of hot alcohol. The mixture was refluxed for four hours, cooled, and filtered. The product, 16.3 Gm. (41.2% of theory), was recrystallized from a mixture of pyridine and alcohol, m. p. 265–266° (total immersion).

Anal.—Calcd. for $C_{18}H_{10}N_4O_4S_2$: S, 15.61%; N, 13.66%. Found: S, 15.44%; N, 13.84%.

15. 2,2′,4,4′-Tetranitrodiphenyl Disulfide.—A modification of the procedure described by Elgersma (7) and Teppema and Sebrell (8) was used. The starting materials were 2,4-dinitrochlorobenzene (53.4 Gm.), alcohol (675 cc.), crystalline $Na_2S.9H_2O$ (32 Gm.), and sulfur (4 Gm.). The product, after washing with water, alcohol, and ether, was almost pure and weighed 43.5 Gm. (83% of theory). After recrystallization from nitrobenzene the product decomposed at 280°. Elgersma (7) reports the decomposition point as 280°.

16. 2,2′,4,4′-Tetra-aminodiphenyl Disulfide.—2,2′,4,4′-Tetranitrodiphenyl disulfide (12 Gm.) was mixed with a solution of 91 Gm. $SnCl_2.2H_2O$ in 95 cc. of concentrated HCl. The mixture was heated on a steam bath for twenty hours, cooled, filtered, and the filtrate diluted with water and made alkaline with 15% NH_4OH. Air was passed through the solution for twenty-four hours and the product (1.5 Gm.) filtered off. Recrystallization from a mixture of benzene and alcohol gave a product melting at 147–148° (total immersion). Muller (9) reported a melting point of 146–147°.

Chemotherapeutic Activity.[1]—Table I indicates the antimalarial activity of some of the compounds prepared.

Compounds numbered CJ-2, CJ-1, CJ-115–2, CJ-601–1, and CJ-3 (Table I) have been tested against tetanus toxin in mice. None of the drugs were effective.

Compounds numbered CJ-2, CJ-1, CJ-106–2, CJ-115–2, CJ-601–1, and SN 8167 have been subjected to standardized Swiss mouse therapeutic tests against influenza virus. None of the drugs have produced any significant cures.

Therapy tests with compound number SN 8167 were conducted on rats infected with Trypanosoma brucei, with intraperitoneal doses ranging from 50 mg. to 75 mg. per rat. No trypanocidal action was detected.

[1] We are indebted to Eli Lilly and Company for permission to quote the results of chemotherapeutic tests and for much helpful discussion.

TABLE I

Name	Number	Antimalarial Activity[b]
5,5′-Diamino-8,8′-diquinolyl disulfide	SN 8167[a]	Q, 1.07 Toxicity LD_{50} 1760 mg./Kg.
5,5′-Diacetamido-8,8′-diquinolyl disulfide	SN 9583	Q, 4.0 Toxicity *LD* 2500 mg./Kg.
5-(*p*-Aminobenzene-*sulfonamido*)-8-chloroquinoline[c]	CJ-1	Inactive 1–50 Gm. Q, 0.16 i Toxicity LD_{50} 2500 mg./Kg.
5-(*p*-Acetamidobenzene-sulfonamido)-8-chloro-quinoline[c]	CJ-2	Inactive 5–200 mg. Q, 0.08 i
5,5′-*bis*(*p*-Acetamido-benzenesulfonamido)-8,8′-diquinolyl disulfide	CJ-5	Inactive 1–50 mg. Q, 0.16 i
8,8′-Diamino-7,7′-diquinolyl sulfide	CJ-105–2	Q, 0.2 i
Sodium-5-amino-quinoline-8-sulfonate[c]	CJ-116–1	Q, 0.2 i
2,2′,4,4′-Tetrachloro-dibenzyl disulfide	CJ-243–1	Inactive 5–100 mg. Q, 0.12 i
3,3′,4,4′-Tetrachloro-dibenzyl disulfide	CJ-244–1	Inactive 5–100 mg. Q, 1.12 i
2,2′-Dichloro-dibenzyl disulfide	CJ-245–1	Inactive 5–100 mg. Q, 0.12 i
4,4′-Dichloro-dibenzyl disulfide	CJ-246–1	Inactive 5–100 mg. Q, 0.12 i
β,β′-Dianthraquinone disulfide	CJ-601–1	Inactive 5–100 mg. Q, 0.16 i

[a] SN = Survey number, an identifying number for compounds which will appear in a forthcoming monograph entitled "A Survey of Antimalarial Drugs, 1941–1945," F. Y. Wiselogle, Editor.

[b] Antimalarial activity as reported by Eli Lilly and Company. The screening tests were obtained with ducks infected with Plasmodium lophurae.

[c] Compounds, the synthesis of which have been previously reported by Christian and Jenkins (2).

SUMMARY

1. The synthesis of a number of new thio and dithio compounds has been described.

2. The antimalarial activity and results of other chemotherapeutic tests of these compounds have been presented.

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The Synthesis of Chemotherapeutic Agents. II.* The Synthesis of Certain Thio and Dithio Compounds†,‡

By JOHN W. WETZEL, DONALD E. WELDTON, JOHN E. CHRISTIAN, GLENN L. JENKINS, and G. BRYANT BACHMAN

In this paper, the second of a series, the continuation of a study of certain thio and dithio compounds related to 5,5′-diacetamido-8,8′-diquinolyl disulfide is described. Of the fourteen sulfur compounds synthesized and characterized, which are described in this paper, twelve are new. Several of these have undergone screening tests for antimalarial activity. Incidental to the syntheses reported new methods are described for the preparation of 2,3- and 4-chloro isomers of 5-nitroquinoline.

THIS IS a report on the continuation of a study of certain thio and dithio compounds the preparation of which were indicated in an effort to produce compounds of higher pharmacological activity and better pharmacological properties than a related compound, 5,5′-diacetamido-8,8′-diquinolyl disulfide, which has been shown to possess antimalarial activity (1).

In this paper 14 additional compounds have been described, some of which have been tested for antimalarial activity.

EXPERIMENTAL

1. **3,3′-Dinitro-4,4′-diquinolyl Sulfide.**—A solution of 12.0 Gm. of $Na_2S.9H_2O$ in 30 cc. of water was diluted with 100 cc. of methanol; 1.60 Gm. of powdered sulfur was added, and the mixture warmed until solution was complete. The resulting solution of Na_2S_2 was cooled to room temperature and added slowly to a well-stirred and cooled solution of 20.8 Gm. of powdered 3-nitro-4-chloroquinoline in 40 cc. of chloroform, keeping the temperature at 20–25°. The mixture was stirred for thirty minutes after all the Na_2S_2 was added and the precipitate of 3,3′-dinitro-4,4′-diquinolyl sulfide filtered off, washed with methanol, stirred with excess dilute NaOH, washed with water, and dried. Yield 19.7 Gm. (96% of theory) of yellow crystals which began to decompose and darken at about 200° and eventually melted with decomposition. No definite melting point could be obtained even after several recrystallizations from toluene.

Anal.—Calcd. for $C_{18}H_{10}N_4O_4S$: N, 14.81%. Found: N, 14.84%.

Comments: It was expected that the above reaction would yield the disulfide. Analysis indicated that the product was the sulfide. This was confirmed by substituting Na_2S for Na_2S_2 in the synthesis.

A mixture of 20.8 Gm. of 3-nitro-4-chloroquinoline in 40 cc. of chloroform was stirred while a solution of 12.0 Gm. of $Na_2S.9H_2O$ in 30 cc. of H_2O and 100 cc. of methanol was added dropwise for ten to fifteen minutes, keeping the temperature below 25°. The mixture was stirred for thirty minutes after all the Na_2S had been added, then filtered. The residue was washed with methanol, suspended in water, and made alkaline with NaOH, filtered, and the residue washed with water and dried. The yield of 3,3′-dinitro-4,4′-diquinolyl sulfide was 17.0–18.0 Gm. (90–95% of theory). The product was identical with the product from the Na_2S_2 reaction.

Anal.—Calcd. for $C_{18}H_{10}N_4O_4S$: N, 14.81%. Found: N, 14.77%.

2. **3-Amino-4-quinolinethiol.**—A mixture of 18.9 Gm. of crude 3,3′-dinitro-4,4′-diquinolyl sulfide, 92 Gm. (10% excess) of $Na_2S.9H_2O$, and 300 cc. of 50% alcohol was stirred and heated slowly to boiling, refluxed for two hours, cooled slightly, slowly acidified with 100 cc. of concentrated HCl, and cooled to room temperature. The 3-amino-4-quinolinethiol hydrochloride was filtered off, washed with 50% alcohol, and suspended in 200 cc. of water. Concentrated NaOH was added until the solution was strongly basic and 1 to 2 Gm. of anhydrous Na_2SO_3 was added and the mixture stirred until the orange thiol had disappeared. The sulfur was filtered off, the filtrate treated with charcoal without heating and filtered again. The clear filtrate was treated, with stirring, with glacial acetic acid, added dropwise until the *p*H fell to about 5. The mixture was allowed to stand for fifteen to thirty minutes and filtered. The crystalline product was washed with water and dried over P_2O_5. Yield 16.2–17.6 Gm. (92–100% of the theory) of orange needles, which began to decompose at about 190° and slowly carbonized. On recrystallization the product was easily oxidized to the disulfide; therefore the crude product was used for further synthesis.

3. **3,3′-Diamino-4,4′-diquinolyl Disulfide.**—A solution of 16.6 Gm. of crude 3-amino-4-quinoline-

* For previous papers in this series see Christian and Jenkins, THIS JOURNAL, 34, 147–[illegible], and 35, [illegible]

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242–244° (total immersion). Attempts to reduce this compound to the corresponding amino compound failed.

Anal.—Calcd. for $C_{18}H_{10}N_4O_4S$: S, 8.47%; N, 14.82%. Found: S, 8.80%; N, 14.97%.

14. 5,5′-Dinitro-1,1′-diisoquinolyl Disulfide.—1-Chloro-5-nitroisoquinoline (20 Gm.) was dissolved in 500 cc. of hot alcohol. To this hot solution was added a warm solution of Na_2S_2, prepared by dissolving 11.1 Gm. $Na_2S.9H_2O$ and 1.5 Gm. sulfur in 110 cc. of hot alcohol. The mixture was refluxed for four hours, cooled, and filtered. The product, 16.3 Gm. (41.2% of theory), was recrystallized from a mixture of pyridine and alcohol, m. p. 265–266° (total immersion).

Anal.—Calcd. for $C_{18}H_{10}N_4O_4S_2$: S, 15.61%; N, 13.66%. Found: S, 15.44%; N, 13.84%.

15. 2,2′,4,4′-Tetranitrodiphenyl Disulfide.—A modification of the procedure described by Elgersma (7) and Teppema and Sebrell (8) was used. The starting materials were 2,4-dinitrochlorobenzene (53.4 Gm.), alcohol (675 cc.), crystalline $Na_2S.9H_2O$ (32 Gm.), and sulfur (4 Gm.). The product, after washing with water, alcohol, and ether, was almost pure and weighed 43.5 Gm. (83% of theory). After recrystallization from nitrobenzene the product decomposed at 280°. Elgersma (7) reports the decomposition point as 280°.

16. 2,2′,4,4′-Tetra-aminodiphenyl Disulfide.—2,2′,4,4′-Tetranitrodiphenyl disulfide (12 Gm.) was mixed with a solution of 91 Gm. $SnCl_2.2H_2O$ in 95 cc. of concentrated HCl. The mixture was heated on a steam bath for twenty hours, cooled, filtered, and the filtrate diluted with water and made alkaline with 15% NH_4OH. Air was passed through the solution for twenty-four hours and the product (1.5 Gm.) filtered off. Recrystallization from a mixture of benzene and alcohol gave a product melting at 147–148° (total immersion). Muller (9) reported a melting point of 146–147°.

Chemotherapeutic Activity.[1]—Table I indicates the antimalarial activity of some of the compounds prepared.

Compounds numbered CJ-2, CJ-1, CJ-115-2, CJ-601-1, and CJ-3 (Table I) have been tested against tetanus toxin in mice. None of the drugs were effective.

Compounds numbered CJ-2, CJ-1, CJ-106-2, CJ-115-2, CJ-601-1, and SN 8167 have been subjected to standardized Swiss mouse therapeutic tests against influenza virus. None of the drugs have produced any significant cures.

Therapy tests with compound number SN 8167 were conducted on rats infected with Trypanosoma brucei, with intraperitoneal doses ranging from 50 mg. to 75 mg. per rat. No trypanocidal action was detected.

[1] We are indebted to Eli Lilly and Company for permission to quote the results of chemotherapeutic tests and for much helpful discussion.

TABLE I

Name	Number	Antimalarial Activity[b]
5,5′-Diamino-8,8′-diquinolyl disulfide	SN 8167[a]	Q, 1.07 Toxicity LD_{50} 1760 mg./Kg.
5,5′-Diacetamido-8,8′-diquinolyl disulfide	SN 9583	Q, 4.0 Toxicity LD 2500 mg./Kg.
5-(*p*-Aminobenzenesulfonamido)-8-chloroquinoline[c]	CJ-1	Inactive 1–50 Gm. Q, 0.16 i Toxicity LD_{50} 2500 mg./Kg.
5-(*p*-Acetamidobenzenesulfonamido)-8-chloroquinoline[c]	CJ-2	Inactive 5–200 mg. Q, 0.08 i
5,5′-*bis*(*p*-Acetamidobenzenesulfonamido)-8,8′-diquinolyl disulfide	CJ-5	Inactive 1–50 mg. Q, 0.16 i
8,8′-Diamino-7,7′-diquinolyl sulfide	CJ-105-2	Q, 0.2 i
Sodium-5-amino-quinoline-8-sulfonate[c]	CJ-116-1	Q, 0.2 i
2,2′,4,4′-Tetrachlorodibenzyl disulfide	CJ-243-1	Inactive 5–100 mg. Q, 0.12 i
3,3′,4,4′-Tetrachlorodibenzyl disulfide	CJ-244-1	Inactive 5–100 mg. Q, 1.12 i
2,2′-Dichloro-dibenzyl disulfide	CJ-245-1	Inactive 5–100 mg. Q, 0.12 i
4,4′-Dichloro-dibenzyl disulfide	CJ-246-1	Inactive 5–100 mg. Q, 0.12 i
β,β′-Dianthraquinone disulfide	CJ-601-1	Inactive 5–100 mg. Q, 0.16 i

[a] SN = Survey number, an identifying number for compounds which will appear in a forthcoming monograph entitled "A Survey of Antimalarial Drugs, 1941–1945," F. Y. Wiselogle, Editor.

[b] Antimalarial activity as reported by Eli Lilly and Company. The screening tests were obtained with ducks infected with Plasmodium lophurae.

[c] Compounds, the synthesis of which have been previously reported by Christian and Jenkins (2).

SUMMARY

1. The synthesis of a number of new thio and dithio compounds has been described.

2. The antimalarial activity and results of other chemotherapeutic tests of these compounds have been presented.

REFERENCES

(1) Report to appear in a forthcoming monograph entitled "A Survey of Antimalarial Drugs, 1941–1945," F. Y. Wiselogle, Editor.
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The Synthesis of Chemotherapeutic Agents. II.* The Synthesis of Certain Thio and Dithio Compounds†,‡

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thiol in 60 cc. of pyridine was stirred and cooled while 12.0 Gm. of solid I_2 was added slowly. The solution became deep red and heat was evolved. Ten grams of Na_2CO_3 dissolved in 100 cc. of water was added slowly, the mixture stirred, allowed to cool to room temperature, filtered and the residue washed well with water. The yellow crystals, after drying at 60°, became orange in color and melted at 123–125°, resolidified to an orange solid and melted at 157°. Heated slowly, the crystals turned orange above 100° and melted at 157°; yield 16.0 Gm. (96% of theory). If recrystallized from alcohol and water the m. p. is raised to 169–171° (decomp.).

Anal.—Calcd. for $C_{18}H_{14}N_4S_2$: N, 16.00%. Found: N, 15.89%.

4. **3-Acetylamino-4-quinolinethiol.**—A solution of 16.7 Gm. of crude 3-amino-4-quinolinethiol in 40 cc. of pyridine was stirred vigorously while 20 cc. (100% excess) of acetic anhydride was added rapidly. The mixture was stirred ten to fifteen minutes, 150 cc. of boiling water added, stirred, and cooled to room temperature. The product was filtered off, washed with water and dried. Yield, 19.9 Gm. (96% of theory). Recrystallization from pyridine yielded pure 3-acetylamino-4-quinolinethiol as yellow needles which darkened about 250° and melted with evolution of gas at about 260–264°.

Anal.—Calcd. $C_{11}H_{10}N_2OS$: N, 12 84. Found: N, 12.83.

5. **5-Nitroquinoline.**—The procedure used for the nitration of quinoline was a combination of that used by Dufton (4) and Fieser and Hershberg (5). The modifications were made in an effort to obtain as large a yield of 5-nitroquinoline as possible.

One hundred grams (0.775 mole) of synthetic quinoline was placed in an evaporating dish cooled with cold water. Sixty-six grams (20% excess) of fuming nitric acid was added dropwise with continuous agitation. The quinoline nitrate was broken up into small pea-sized particles and divided into ten equal portions. These portions were added to 100 cc. of concentrated H_2SO_4 which was contained in a suitable flask cooled in an ice bath. After each portion of the nitrate was added, 10 cc. of 20% oleum was stirred in controlling the temperature of the mixture between 10° and 20°. After all the reactants were in the flask the mixture was stirred for one hour, keeping the temperature at 10–20°. The liquid was poured into about 800 Gm. of crushed ice and the solution neutralized with 500–600 cc. of NH_4OH. The solid which formed on cooling was separated and dissolved in 670 cc. of hot dilute HNO_3 (d. 1.12). The solution was cooled and a yellow solid crystallized. This solid was dissolved in hot water and neutralized with NH_4OH. An oil separated and crystallized on cooling. The yield of pure 5-nitroquinoline was 50 Gm. (36.5% of theory). The nitrate melted at 186–186.5°, and the free base at 71–72°.

6. **Perphthalic Acid.**—Perphthalic acid was prepared according to the procedure described by Cooper (6), treating phthalic anhydride with H_2O_2 in NaOH solution. The peracid content determined by iodometric titration corresponded to a 70% yield.

7. **5-Nitroquinoline N-Oxide Phthalate.**—Sixty grams (0.35 mole) of 5-nitroquinoline was dissolved in 700 cc. of ether which contained 95 Gm. (0.52 mole) of perphthalic acid and the mixture was allowed to stand for twenty-four hours at 15°. A yellow precipitate gradually formed, giving a quantitative yield of crude product.

8. **5-Nitroquinoline N-Oxide.**—A solution of one liter of water and 85 Gm. of sodium carbonate was stirred vigorously as 152.6 Gm. of powdered 5-nitroquinoline N-oxide phthalate was added slowly. The mixture was stirred for forty-five minutes and then the orange-yellow solid was filtered out and washed with ice water until the washings were neutral and colorless. The yield was 67% of crude product. Recrystallization from toluene gave a product melting at 163–164°.

Anal.—Calcd. for $C_9H_6N_2O_3$: C, 56.85; H, 3.18. Found: C, 56.86; H, 3.26.

9. **2-Chloro-5-nitroquinoline, 3-Chloro-5-nitroquinoline, and 4-Chloro-5-nitroquinoline.**—Fifty grams (0.26 mole) of finely powdered 5-nitroquinoline N-oxide was dissolved slowly in 225 cc. of phosphorus oxychloride contained in a cooled flask under reflux. The flask was transferred to an oil bath heated to 60° and the temperature slowly raised to 105–115° for two hours. The excess $POCl_3$ was removed by vacuum distillation and the residue poured onto 500 Gm. of crushed ice. The mixture was stirred for two hours at 5–10° to hydrolyze the $POCl_3$. The insoluble material, 2-chloro-5-nitroquinoline, was filtered off and washed with cold water until the washings were neutral. The washings were added to the mother liquor and the total volume made up to one liter. A small amount of a solid mixture of 2- and 3-chloro-5-nitroquinolines separated and was filtered off, and the filtrate diluted to two liters total volume. More solid, a mixture of 3- and 4-chloro-5-nitroquinolines, separated and was filtered off. The filtrate was kept cold while 100 cc. of concentrated ammonium hydroxide was added dropwise. A mixture of 3- and 4-chloro-5-nitroquinolines separated. The filtrate was allowed to stand in the cold and filtered to remove a mixture of 4-chloro-5-nitroquinoline and an alkali soluble impurity.

The fractions were treated chemically to separate the isomers. 2-Chloro-5-nitroquinoline was separated from 3-chloro-5-nitroquinoline by virtue of the insolubility of the 2-isomer in 6 *N* HCl. The mixture was dissolved in concentrated HCl and then diluted with an equal volume of water. The 2-isomer separated from this solution.

3-Chloro- and 4-chloro-5-nitroquinolines were separated through the insolubility of the 3-isomer in 3 *N* HCl. The mixture was stirred well with 3 *N* HCl, and the insoluble 3-isomer filtered off. The filtrate was slowly neutralized in the cold with NH_4OH to precipitate 4-chloro-5-nitroquinoline.

The identity of the compounds was established as follows:

2-Chloro-5-nitroquinoline obtained in 44.5% yield was recrystallized from methanol and melted at 133–134°, literature value 129° (2, 3). A mixed melting point with material prepared at the University of Virginia (O. S. R. D.) showed no depression. Hydrolysis of the compound with concentrated HCl gave 2-hydroxy-5-nitroquinoline, m. p. 301–303°, literature value 298–299° (2, 3).

3-Chloro-5-nitroquinoline obtained in 13.1% yield was recrystallized from ethanol and melted at 128–129°, literature value 127–128° (7). This compound could not be hydrolyzed on prolonged treating with concentrated HCl.

4-Chloro-5-nitroquinoline obtained in 9.4% yield was recrystallized from methanol, m. p. 149–150°.

Anal.—Calcd. for $C_9H_5N_2O_2Cl$: C, 51.82; H, 2.42. Found: C, 51.76; H, 2.45.

The chloro compound was hydrolyzed by heating with concentrated HCl to give 4-hydroxy-5-nitroquinoline which had an indefinite decomposition point of 335–340°.

Anal.—Calcd. for $C_9H_6N_2O_3$: C, 56.84; H, 3.18. Found: C, 56.86; H, 3.40.

10. 5,5′-Dinitro-2,2′-diquinolyl Sulfide.—The starting material, 2-chloro-5-nitroquinoline, used in this synthesis was obtained from two sources. A 45-Gm. sample was obtained from O. S. R. D. It was purified by recrystallization from alcohol until the product melted at 127–129°. The melting point for the pure material has been reported as 130° (2, 3). A second portion was prepared by the chlorination of the N-oxide of 5-nitroquinoline.

A solution of 5.2 Gm. of 2-chloro-5-nitroquinoline in 75 cc. of alcohol was refluxed and stirred. A solution of sodium disulfide (prepared from 3 Gm. of $Na_2S.9H_2O$ and 0.4 Gm. of sulfur in 25 cc. of alcohol and 6 cc. of water) was added slowly during forty-five minutes. The solution was allowed to reflux for eleven hours and cooled in an ice chest overnight. The yellow precipitate was filtered off, rinsed with alcohol, triturated with dilute NaOH, washed with water and alcohol and dried. Two and two-tenths grams of sulfide was obtained which on recrystallization from pyridine melted with decomposition at 260–261°. The NaOH filtrate was acidified with glacial acetic acid yielding an orange precipitate of 5-nitro-2-quinolinethiol; m. p. 234–236°. Additional quantities of these products were obtained by working up the original filtrate. The combined yield of crude 5,5′-dinitro-2,2′-diquinolyl sulfide was 3.7 Gm. (73% of theory). The total 5-nitro-2-quinolinethiol obtained was 0.9 Gm. (21% of theory).

Anal.—Calcd. for $C_{18}H_{10}N_4O_4S$: C, 57.14; H, 2.67. Found: C, 57.12; H, 2.77.

11. 5 - Acetamido - 2 - chloroquinoline.—Eight grams of 2-chloro-5-nitroquinoline was dissolved in 140 cc. of glacial acetic acid and 20 cc. of acetic anhydride (50% excess). Approximately 1 Gm. of Raney nickel catalyst was added and the mixture reduced at low pressure and room temperature. The reaction mixture was filtered, treated with Norite, and filtered again. The filtrate was slowly neutralized with NH_4OH as it was stirred in an ice bath. A white solid gradually precipitated as the solution approached neutrality. The solid was filtered and washed with water. The material was recrystallized from ethanol to give a product, m. p. 214–215°, yield 75% of theory.

Anal.—Calcd. for $C_{11}H_9N_2OCl$: C, 59.93%; H, 4.11%. Found: C, 59.92%; H, 4.38%.

12. 5-Acetamido-2-quinolinethiol.—Two grams 5-acetamido-2-chloroquinoline was dissolved in 35 cc. of ethanol by heating to reflux. To this hot solution an alcoholic solution of Na_2S_2 was added dropwise over a period of five hours. The Na_2S_2 solution was prepared by dissolving 1.07 Gm. of $Na_2S.9H_2O$ in 10.2 cc. of ethanol and 3.4 cc. of water and then adding 0.14 Gm. of sulfur. The mixture was refluxed with stirring for twenty hours. The yellow solid was filtered off and dissolved in 10% NaOH. The alkaline solution was treated with Norite and the yellow solid reprecipitated with acetic acid; yield 51.5% of theory. Recrystallization from alcohol yielded a product melting at 274–278° with decomposition.

Anal.—Calcd. for $C_{11}H_{10}N_2OS$: S, 14.69%. Found: S, 14.72%.

13. Bis(6 - methoxy - 2 - methyl - 7,8,9,10 - tetrahydro-4-benzo(h)quinolyl) Sulfide.—To 100 cc. of warm alcohol, 5.23 Gm. of 4-chloro-6-methoxy-2-methyl-7,8,9,10-tetrahydrobenzo(h)quinoline was added. Complete solution occurred at 60° after which a warm alcoholic solution of Na_2S_2 (prepared by dissolving 4.72 Gm. $Na_2S.9H_2O$ and 0.626 Gm. powdered S in 40 cc. of 95% alcohol) was added with stirring. The solution was refluxed seventeen hours, cooled, diluted with water to dissolve the NaCl, and filtered; yield 4.8 Gm. (89% of theory). Recrystallization from dioxan gave a product melting at 200–209°; from toluene the melting point was 211–218°.

Anal.—Calcd. for $C_{30}H_{32}O_2N_2S$: S, 6.62%. Found: S, 7.01%.

14. 3-Amino-4-quinolinesulfonic Acid.—A solution of 4.4 Gm. of 3-amino-4-quinolinethiol in 45 cc. of 50% methanol containing 1.1 Gm. (10% excess) of NaOH was cooled and stirred while 9.4 Gm. (10% excess) of 30% H_2O_2 was added dropwise keeping the temperature at 30–35°. Stirring and cooling were continued until the precipitate which formed had redissolved, and the solution then refluxed from ten to fifteen minutes. It was then acidified with 20 cc. of 50% H_2SO_4 and allowed to cool to room temperature. The yellow precipitate was filtered off, washed with methanol and dried. The product was purified by reprecipitation. The yield of pure light yellow 3-amino-4-quinolinesulfonic acid was 4.0 Gm. (72% of theory). The product has no definite melting point but darkens at about 240°, and melts with decomposition between 250–265°.

thiol in 60 cc. of pyridine was stirred and cooled while 12.0 Gm. of solid I_2 was added slowly. The solution became deep red and heat was evolved. Ten grams of Na_2CO_3 dissolved in 100 cc. of water was added slowly, the mixture stirred, allowed to cool to room temperature, filtered and the residue washed well with water. The yellow crystals, after drying at 60°, became orange in color and melted at 123–125°, resolidified to an orange solid and melted at 157°. Heated slowly, the crystals turned orange above 100° and melted at 157°; yield 16.0 Gm. (96% of theory). If recrystallized from alcohol and water the m. p. is raised to 169–171° (decomp.).

Anal.—Calcd. for $C_{18}H_{14}N_4S_2$: N, 16.00%. Found: N, 15.89%.

4. **3-Acetylamino-4-quinolinethiol.**—A solution of 16.7 Gm. of crude 3-amino-4-quinolinethiol in 40 cc. of pyridine was stirred vigorously while 20 cc. (100% excess) of acetic anhydride was added rapidly. The mixture was stirred ten to fifteen minutes, 150 cc. of boiling water added, stirred, and cooled to room temperature. The product was filtered off, washed with water and dried. Yield, 19.9 Gm. (96% of theory). Recrystallization from pyridine yielded pure 3-acetylamino-4-quinolinethiol as yellow needles which darkened about 250° and melted with evolution of gas at about 260–264°.

Anal.—Calcd. $C_{11}H_{10}N_2OS$: N, 12 84. Found: N, 12 83.

5. **5-Nitroquinoline.**—The procedure used for the nitration of quinoline was a combination of that used by Dufton (4) and Fieser and Hershberg (5). The modifications were made in an effort to obtain as large a yield of 5-nitroquinoline as possible.

One hundred grams (0.775 mole) of synthetic quinoline was placed in an evaporating dish cooled with cold water. Sixty-six grams (20% excess) of fuming nitric acid was added dropwise with continuous agitation. The quinoline nitrate was broken up into small pea-sized particles and divided into ten equal portions. These portions were added to 100 cc. of concentrated H_2SO_4 which was contained in a suitable flask cooled in an ice bath. After each portion of the nitrate was added, 10 cc. of 20% oleum was stirred in controlling the temperature of the mixture between 10° and 20°. After all the reactants were in the flask the mixture was stirred for one hour, keeping the temperature at 10–20°. The liquid was poured into about 800 Gm. of crushed ice and the solution neutralized with 500–600 cc. of NH_4OH. The solid which formed on cooling was separated and dissolved in 670 cc. of hot dilute HNO_3 (d. 1.12). The solution was cooled and a yellow solid crystallized. This solid was dissolved in hot water and neutralized with NH_4OH. An oil separated and crystallized on cooling. The yield of pure 5-nitroquinoline was 50 Gm. (36.5% of theory). The nitrate melted at 186–186.5°, and the free base at 71–72°.

6. **Perphthalic Acid.**—Perphthalic acid was prepared according to the procedure described by Cooper (6), treating phthalic anhydride with H_2O_2 in NaOH solution. The peracid content determined by iodometric titration corresponded to a 70% yield.

7. **5-Nitroquinoline N-Oxide Phthalate.**—Sixty grams (0.35 mole) of 5-nitroquinoline was dissolved in 700 cc. of ether which contained 95 Gm. (0.52 mole) of perphthalic acid and the mixture was allowed to stand for twenty-four hours at 15°. A yellow precipitate gradually formed, giving a quantitative yield of crude product.

8. **5-Nitroquinoline N-Oxide.**—A solution of one liter of water and 85 Gm. of sodium carbonate was stirred vigorously as 152.6 Gm. of powdered 5-nitroquinoline N-oxide phthalate was added slowly. The mixture was stirred for forty-five minutes and then the orange-yellow solid was filtered out and washed with ice water until the washings were neutral and colorless. The yield was 67% of crude product. Recrystallization from toluene gave a product melting at 163–164°.

Anal.—Calcd. for $C_9H_6N_2O_3$: C, 56.85; H, 3.18. Found: C, 56.86; H, 3.26.

9. **2-Chloro-5-nitroquinoline, 3-Chloro-5-nitroquinoline, and 4-Chloro-5-nitroquinoline.**—Fifty grams (0.26 mole) of finely powdered 5-nitroquinoline N-oxide was dissolved slowly in 225 cc. of phosphorus oxychloride contained in a cooled flask under reflux. The flask was transferred to an oil bath heated to 60° and the temperature slowly raised to 105–115° for two hours. The excess $POCl_3$ was removed by vacuum distillation and the residue poured onto 500 Gm. of crushed ice. The mixture was stirred for two hours at 5–10° to hydrolyze the $POCl_3$. The insoluble material, 2-chloro-5-nitroquinoline, was filtered off and washed with cold water until the washings were neutral. The washings were added to the mother liquor and the total volume made up to one liter. A small amount of a solid mixture of 2- and 3-chloro-5-nitroquinolines separated and was filtered off, and the filtrate diluted to two liters total volume. More solid, a mixture of 3- and 4-chloro-5-nitroquinolines, separated and was filtered off. The filtrate was kept cold while 100 cc. of concentrated ammonium hydroxide was added dropwise. A mixture of 3- and 4-chloro-5-nitroquinolines separated. The filtrate was allowed to stand in the cold and filtered to remove a mixture of 4-chloro-5-nitroquinoline and an alkali soluble impurity.

The fractions were treated chemically to separate the isomers. 2-Chloro-5-nitroquinoline was separated from 3-chloro-5-nitroquinoline by virtue of the insolubility of the 2-isomer in 6 *N* HCl. The mixture was dissolved in concentrated HCl and then diluted with an equal volume of water. The 2-isomer separated from this solution.

3-Chloro- and 4-chloro-5-nitroquinolines were separated through the insolubility of the 3-isomer in 3 *N* HCl. The mixture was stirred well with 3 *N* HCl, and the insoluble 3-isomer filtered off. The filtrate was slowly neutralized in the cold with NH_4OH to precipitate 4-chloro-5-nitroquinoline.

The identity of the compounds was established as follows:

2-Chloro-5-nitroquinoline obtained in 44.5% yield was recrystallized from methanol and melted at 133–134°, literature value 129° (2, 3). A mixed melting point with material prepared at the University of Virginia (O. S. R. D.) showed no depression. Hydrolysis of the compound with concentrated HCl gave 2-hydroxy-5-nitroquinoline, m. p. 301–303°, literature value 298–299° (2, 3).

3-Chloro-5-nitroquinoline obtained in 13.1% yield was recrystallized from ethanol and melted at 128–129°, literature value 127–128° (7). This compound could not be hydrolyzed on prolonged treating with concentrated HCl.

4-Chloro-5-nitroquinoline obtained in 9.4% yield was recrystallized from methanol, m. p. 149–150°.

Anal.—Calcd. for $C_9H_5N_2O_2Cl$: C, 51.82; H, 2.42. Found: C, 51.76; H, 2.45.

The chloro compound was hydrolyzed by heating with concentrated HCl to give 4-hydroxy-5-nitroquinoline which had an indefinite decomposition point of 335–340°.

Anal.—Calcd. for $C_9H_6N_2O_3$: C, 56.84; H, 3.18. Found: C, 56.86; H, 3.40.

10. 5,5′-Dinitro-2,2′-diquinolyl Sulfide.—The starting material, 2-chloro-5-nitroquinoline, used in this synthesis was obtained from two sources. A 45-Gm. sample was obtained from O. S. R. D. It was purified by recrystallization from alcohol until the product melted at 127–129°. The melting point for the pure material has been reported as 130° (2, 3). A second portion was prepared by the chlorination of the N-oxide of 5-nitroquinoline.

A solution of 5.2 Gm. of 2-chloro-5-nitroquinoline in 75 cc. of alcohol was refluxed and stirred. A solution of sodium disulfide (prepared from 3 Gm. of $Na_2S.9H_2O$ and 0.4 Gm. of sulfur in 25 cc. of alcohol and 6 cc. of water) was added slowly during forty-five minutes. The solution was allowed to reflux for eleven hours and cooled in an ice chest overnight. The yellow precipitate was filtered off, rinsed with alcohol, triturated with dilute NaOH, washed with water and alcohol and dried. Two and two-tenths grams of sulfide was obtained which on recrystallization from pyridine melted with decomposition at 260–261°. The NaOH filtrate was acidified with glacial acetic acid yielding an orange precipitate of 5-nitro-2-quinolinethiol; m. p. 234–236°. Additional quantities of these products were obtained by working up the original filtrate. The combined yield of crude 5,5′-dinitro-2,2′-diquinolyl sulfide was 3.7 Gm. (73% of theory). The total 5-nitro-2-quinolinethiol obtained was 0.9 Gm. (21% of theory).

Anal.—Calcd. for $C_{18}H_{10}N_4O_4S$: C, 57.14; H, 2.67. Found: C, 57.12; H, 2.77.

11. 5 - Acetamido - 2 - chloroquinoline.—Eight grams of 2-chloro-5-nitroquinoline was dissolved in 140 cc. of glacial acetic acid and 20 cc. of acetic anhydride (50% excess). Approximately 1 Gm. of Raney nickel catalyst was added and the mixture reduced at low pressure and room temperature. The reaction mixture was filtered, treated with Norite, and filtered again. The filtrate was slowly neutralized with NH_4OH as it was stirred in an ice bath. A white solid gradually precipitated as the solution approached neutrality. The solid was filtered and washed with water. The material was recrystallized from ethanol to give a product, m. p. 214–215°, yield 75% of theory.

Anal.—Calcd. for $C_{11}H_9N_2OCl$: C, 59.93%; H, 4.11%. Found: C, 59.92%; H, 4.38%.

12. 5-Acetamido-2-quinolinethiol.—Two grams 5-acetamido-2-chloroquinoline was dissolved in 35 cc. of ethanol by heating to reflux. To this hot solution an alcoholic solution of Na_2S_2 was added dropwise over a period of five hours. The Na_2S_2 solution was prepared by dissolving 1.07 Gm. of $Na_2S.9H_2O$ in 10.2 cc. of ethanol and 3.4 cc. of water and then adding 0.14 Gm. of sulfur. The mixture was refluxed with stirring for twenty hours. The yellow solid was filtered off and dissolved in 10% NaOH. The alkaline solution was treated with Norite and the yellow solid reprecipitated with acetic acid; yield 51.5% of theory. Recrystallization from alcohol yielded a product melting at 274–278° with decomposition.

Anal.—Calcd. for $C_{11}H_{10}N_2OS$: S, 14.69%. Found: S, 14.72%.

13. Bis(6 - methoxy - 2 - methyl - 7,8,9,10 - tetrahydro-4-benzo(h)quinolyl) Sulfide.—To 100 cc. of warm alcohol, 5.23 Gm. of 4-chloro-6-methoxy-2-methyl-7,8,9,10-tetrahydrobenzo(h)quinoline was added. Complete solution occurred at 60° after which a warm alcoholic solution of Na_2S_2 (prepared by dissolving 4.72 Gm. $Na_2S.9H_2O$ and 0.626 Gm. powdered S in 40 cc. of 95% alcohol) was added with stirring. The solution was refluxed seventeen hours, cooled, diluted with water to dissolve the NaCl, and filtered; yield 4.8 Gm. (89% of theory). Recrystallization from dioxan gave a product melting at 200–209°; from toluene the melting point was 211–218°.

Anal.—Calcd. for $C_{30}H_{32}O_2N_2S$: S, 6.62%. Found: S, 7.01%.

14. 3-Amino-4-quinolinesulfonic Acid.—A solution of 4.4 Gm. of 3-amino-4-quinolinethiol in 45 cc. of 50% methanol containing 1.1 Gm. (10% excess) of NaOH was cooled and stirred while 9.4 Gm. (10% excess) of 30% H_2O_2 was added dropwise keeping the temperature at 30–35°. Stirring and cooling were continued until the precipitate which formed had redissolved, and the solution then refluxed from ten to fifteen minutes. It was then acidified with 20 cc. of 50% H_2SO_4 and allowed to cool to room temperature. The yellow precipitate was filtered off, washed with methanol and dried. The product was purified by reprecipitation. The yield of pure light yellow 3-amino-4-quinolinesulfonic acid was 4.0 Gm. (72% of theory). The product has no definite melting point but darkens at about 240°, and melts with decomposition between 250–265°.

Anal.—Calcd. for $C_5H_5N_2SO_3$: S, 14.28%. Found: S, 14.33%.

SUMMARY

1. Twelve new compounds have been synthesized in an effort to find compounds with desirable chemotherapeutic properties. A new method is described for the preparation of the 2, 3 and 4-chloro-isomers of 5-nitroquinoline.

2. Sufficient quantities of these compounds have been prepared for pharmacological testing.

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The Synthesis of Chemotherapeutic Agents.* III. The Synthesis of Certain Thio Compounds†,‡

By YUEN-FU CHENG, JOHN E. CHRISTIAN, and GLENN L. JENKINS

Five new sulfa compounds possessing possible medicinal value have been synthesized and characterized.

SINCE 5,5′-diamino-8,8′-diquinolyl disulfide and some of its derivatives have been synthesized (1) and investigation has revealed possible chemotherapeutic value for these compounds (2), it is believed that 5,5′-diamino-8,8′-diquinolyl sulfide and its derivatives may have some therapeutic applications. The objective of the project reported here is the synthesis of 5,5′-diamino-8,8′-diquinolyl sulfide and some of its derivatives, so that they might be tested.

The starting material used in this synthesis was *o*-chloroaniline, from which 8-chloroquinolyl, 5-nitro-8-chloroquinoline, and 5,5′-dinitro-8,8′-diquinolyl sulfide have been made by using the procedures described previously (3–6).

The synthesis of 5,5′-diamino-8,8′-diquinolyl sulfide was accomplished when stannous chloride and hydrochloric acid were used as reducing agents to reduce 5,5′-dinitro-8,8′-diquinolyl sulfide. From 5,5′-diamino-8,8′-diquinolyl sulfide, 5,5′-diacetylamido-8,8′-diquinolyl sulfide was prepared by using acetic anhydride and anhydrous sodium acetate; 5,5′-diformylamido-8,8′-diquinolyl sulfide was synthesized by treatment with strong formic acid.

When 5,5′-diamino-8,8′-diquinolyl sulfide was condensed with two molecules of *p*-acetamidobenzene sulfonyl chloride, 5,5′-*bis*-(*p*-acetamidobenzene sulfonamido)-8,8′-diquinolyl sulfide was obtained.

EXPERIMENTAL

1. **8-Chloroquinoline.**—The procedures used were those described by Urist (3), Christian (4), and Fourneau, *et al.* (5). The yield obtained was 67%.

2. **5-Nitro-8-chloroquinoline.**—The procedures used were essentially those of Christian (4) and Fourneau, *et al.* (5). The yield obtained was 81% and the white needle crystals melted at 144.8°. [Urist (3) reported 145°; Fourneau (5) reported 145°.]

3. **5,5′-Dinitro-8,8′-diquinolyl Sulfide.**—By using the procedure described by Surrey and Lindwall (6), a 90% yield was obtained. The product melted at 288°. [Surrey and Lindwall (6) reported m. p. 288.5–290°.]

4. **5,5′-Dinitro-8,8′-diquinolyl Sulfone.**—The procedure used was essentially that described by Surrey and Lindwall (6). Greenish yellow crystals were obtained; m. p. 259°. [Surrey and Lindwall (6) reported m. p. 260°.] The yield was 55.5%.

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* For previous papers in this series see THIS JOURNAL, 34, 147(1945); 35, 328(1946); 35, 331(1946).

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‡ The work described in this paper was done in collaboration with Eli Lilly and Company and the Committee on Medical Research of the Office of Scientific Research and Development.

5. **5,5′ - Diamino - 8,8′ - diquinolyl Sulfide.**—A solution of 25 Gm. of stannous chloride in 50 cc. of concentrated HCl was slowly added with continuous stirring to a cooled solution of 5 Gm. of 5,5′-dinitro-8,8′-diquinolyl sulfide dissolved in 100 cc. of concentrated HCl. The yellow tin precipitate appeared gradually until the reaction was completed. The mixture was cooled in an ice bath about one hour, the precipitate was collected, dried, dissolved in about 60 cc. of 20% NaOH solution, and then cooled. The mixture was filtered and the yellow precipitate was washed with two 10-cc. portions of 20% NaOH solution, with ammonium hydroxide, and then with distilled water. The washed precipitate was dried, dissolved in acetone, and decolorized with activated charcoal. The acetone solution was concentrated and diluted with water to cause crystallization. The product consisted of yellow needle crystals which melted at 242° (decomp.). If the crystals were purified directly from acetone, they melted at 248°. The yield was 2.7 Gm. (64%).

Anal.—Calcd. for $C_{18}H_{14}N_4S$: N, 17.61%; S, 10.06%. Found: N, 17.11%; S, 9.58%.

6. **The Hydrochloride of 5,5′-Diamino-8,8′-diquinolyl Sulfide.**—The initial procedure was like the preparation of 5,5′-diamino-8,8′-diquinolyl sulfide. The tin complex was destroyed by adding 40% NaOH until the mixture was slightly acid; after stirring about half an hour the precipitate which formed was collected. The precipitate was purified by recrystallization from methanol. Orange needle crystals were obtained; m. p. 237.5–238.5°. The yield was 22%.

Anal.—Calcd. for $C_{18}H_{16}N_4SCl_2$: N, 14.32%; S, 8.18%. Found: N, 14.69%; S, 8.22%.

7. **5,5′-Diacetylamino-8,8′-diquinolyl Sulfide.**—Dissolve 0.25 Gm. of 5,5′-diamino-8,8′-diquinolyl sulfide in 30 cc. of acetic anhydride, add 0.25 Gm. of anhydrous sodium acetate, and reflux on an oil bath for three hours. After cooling, the clear solution was poured into 100 cc. of ice water. Sodium carbonate solution was slowly added with continuous stirring; a gray precipitate appeared while the solution was still acid. The mixture was placed in an ice bath for about two hours, the precipitate was collected, dried, and purified by crystallization from a water-alcohol mixture. The gray crude crystals melted at 233–236°. Pure white crystals were obtained by recrystallization from a water-acetone mixture; m. p. 244°. The yield was 0.2 Gm. (63%).

Anal.—Calcd. for $C_{22}H_{18}N_4O_2S$: N, 13.93%; S, 7.96%. Found: N, 13.11%; S, 7.52%.

8. **5,5′-Diformylamido-8,8′-diquinolyl Sulfide.**—One gram of 5,5′-diamino-8,8′-diquinolyl sulfide was dissolved in 20 cc. of formic acid (sp. gr. 1.2). The mixture was refluxed for two hours. After cooling, the reacted mixture was poured into 100 cc. of ice water. The red acidic solution was treated with a saturated sodium carbonate solution almost to the neutral point; an orange precipitate was obtained. The yield was 0.68 Gm. (58%), m. p. 277°. The product was purified further by recrystallization from acetone several times, m. p. 297° (decomp.).

Anal.—Calcd. for $C_{23}H_{14}N_4O_2S$: N, 8.56%; S, 14.97%. Found: N, 8.56%; S, 14.96%.

9. **5,5′-Bis-(*p*-acetamidobenzene Sulfonamido)-8,8′-Diquinolyl Sulfide.**—One gram of 5,5′-diamino-8,8′-diquinolyl sulfide was dissolved in 20 cc. of dry pyridine, and 1.6 Gm. of *p*-acetamidobenzenesulfonylchloride and a small amount of copper-bronze catalyst were added. The mixture, which was contained in a flask attached to a condenser fitted with a calcium chloride tube, was heated on a steam bath for one hour. After cooling, the mixture was poured into 500 cc. of water and placed in an ice bath for about two hours. The precipitate was collected and recrystallized from acetone. When the substance first precipitated in the concentrated acetone solution, yellow crystals formed which changed to an orange crystalline substance after filtration. The yield was 0.9 Gm. (40%), m. p. 259°. When purified further by recrystallization from acetone, white crystals m. p. 263° (decomp.), were obtained.

Anal.—Calcd. for $C_{34}H_{28}N_6O_6S_3$: N, 11.80%; S, 13.48%. Found: N, 11.42; S, 12.86%.

SUMMARY

1. Five new quinoline derivatives possessing possible medicinal value have been synthesized, namely:

(*a*) 5,5′-Diamino-8,8′-diquinolyl sulfide.

(*b*) The hydrochloride of 5,5′-diamino-8,8′-diquinolyl sulfide.

(*c*) 5,5′-Diacetylamido - 8,8′ - diquinolyl sulfide.

(*d*) 5,5′ - Diformylamido-8,8′-diquinolyl sulfide.

(*e*) 5,5′-Bis-(*p*-acetamidobenzene sulfonamido)-8,8′-diquinolyl sulfide.

2. Unfortunately these quinoline derivatives have not been investigated for possible medicinal use; however, sufficient quantities have been prepared for bacteriological and pharmacological testing.

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Quantitative Determination of Saccharin*

By HAROLD ROSENBLUM and LEE MILDWORM

A convenient and rapid method for the assay of saccharin and saccharin sodium in tablets is described. The assay is effected by extracting the saccharin with a mixture of chloroform and ether and titrating the extracted saccharin with $N/10$ sodium hydroxide.

ALTHOUGH the U. S. P. XII method for the determination of saccharin in saccharin sodium tablets has been successfully employed, the analysts have always been aware that the method does not isolate the principal ingredient and that the results are dependent on the completeness of the hydrolysis of the NH group to NH_3. Furthermore, in order to insure that this method be workable, a test for ammonia in saccharin, saccharin sodium, and tablets of saccharin sodium is included in the Pharmacopœia. However, no assurance is given that nitrogen containing substances which slowly form ammonia during hydrolysis do not interfere with the assay.

EXPERIMENTAL

In order to circumvent interference from other NH_3 liberating compounds, the proposed method was investigated and found to be reliable and rapid for the determination of saccharin and saccharin sodium in tablet mixtures.

The chemical structure of saccharin is such as to give a strong acid reaction in aqueous solution. Attempts to titrate the solution using phenolphthalein T. S. as indicator gave excellent results. One-half gram of material accurately weighed and dissolved in 75 cc. of water by warming and cooling showed 99.5% saccharin by titration. Furthermore, saccharin was found to be appreciably soluble in a mixture of chloroform and ether (1:1). From these two established facts, the following procedure was evolved for tablet mixtures. Results are given in Table I with a comparison of values obtained with the U. S. P. XII procedure.

Place a sufficient number of tablets equivalent to 1 Gm. of saccharin in a 100-cc. volumetric flask, and add 50 cc. H_2O and 10 cc. N NaOH. Agitate until the tablets have disintegrated. Dilute to volume with water and filter through a dry filter into a dry flask, rejecting the first 20 cc. of filtrate. Transfer 25 cc. of the subsequent filtrate to a separatory funnel, acidify with dilute HCl, and extract the saccharin with 6 x 25-cc. portions of a mixture of chloroform and ether (1:1). Filter the combined extract through a pledget of cotton into a beaker and evaporate to dryness on a steam bath with the aid of a current of air. The residue obtained after evaporation of the solvents is taken up with 75 cc. of boiling water, the solution cooled to room temperature and titrated with $N/10$ NaOH, using phenolphthalein T. S. as indicator.

1 cc. of $0.1N$ NaOH = 0.01832 Gm. saccharin ($C_7H_5O_3NS$)

1 cc. of $0.1N$ NaOH = 0.02413 Gm. saccharin sodium ($C_7H_4O_3NSNa{\cdot}2H_2O$)

DISCUSSION

The interference of stearates in the proposed method was investigated and found to be negligible. In the initial filtration, it was found that practically all of the stearates are removed. Stearic acid if present reacts with sodium hydroxide in the cold too slowly to come through in the filtrate. Tablets containing stearic acid as much as 5 per cent of the weight of the tablet showed little or no stearate interference. The extracted saccharin evaporated to dryness and dried to constant weight at 90° showed approximately a 1 per cent higher assay value than by titration. Furthermore, filtration of the cooled aqueous solution of the extracted saccharin removes all insoluble organic acids that may be present.

TABLE I

Sample	Per Cent Based on Labeled Amount: Proposed Method	U. S. P. XII Method
Saccharin Powder	99.50%	99.4%
Special Granulation (50%)	102.2%	103.6%
Saccharin Tablets, 1/4 gr.	98.8%	99.6%
Saccharin Tablets, 1/2 gr.	97.2%	98.0%
	95.6%	95.6%
Saccharin Tablets, 1 gr.	96.0%	95.7%
Saccharin Sodium Tablets, 1 gr.	103.9%	103.2%

SUMMARY

A rapid, accurate determination of saccharin in saccharin sodium and in tablet mixtures is presented.

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Evaluation of Totaquine in Treatment of Malaria in an Endemic Malarious Area*,†

By O. W. YEAGER,‡ R. F. REIDER,‡ and G. E. McDANIEL§

A PROGRAM for the purpose of evaluating the usefulness and toxicity of totaquine[1] as an agent in the treatment of naturally occurring malaria was instituted at the Malaria Field Research Station in Manning, S. C. The study which began on June 15, 1945, and extended through November, 1945, was carried on under field conditions in conjunction with other studies on the malarial disease and infections prevalent in the southern portion of Clarendon County, S. C.

Preliminary planning revealed the necessity for recognition of the usual limitations of a drug study carried out under field conditions. Therefore a complete appraisal of all factors involved in drug evaluations was not attempted. The primary objective of this study was simply to ascertain the clinical usefulness and relative toxicity of totaquine as compared with some other established and generally used antimalarial drug with a known degree of toxicity.

Quinine sulfate was chosen as the control drug because it is most frequently used by local physicians throughout the study area in the treatment of malaria, and is the chief ingredient of proprietary antimalarial drugs used by the people in self-medication for the treatment of symptoms alleged to be due to malaria. Quinine sulfate has been used as an antimalarial drug in the area for many years and is most often considered by the people to be the most acceptable drug.

The procedure of study consisted of the treatment at random of cases of clinical malaria with totaquine and quinine sulfate. The schedule of dosage was based on recommendations of the U. S. Army Medical Department (1). The adult therapeutic dose used for both drugs was 1 Gm. orally 3 times daily for two days and then 2 Gm. orally daily for five days. Proportionate dosages were utilized for children, depending upon weight and apparent age. No other drugs were prescribed. Each treated case was followed up to determine the relative acceptability of the two drugs, the occurrence of toxic symptoms, and to make a clinical appraisal of the relative effectiveness of the drugs as therapeutic antimalarial agents. Since this study was operated under natural field conditions, no extensive laboratory investigations were carried out.

The diagnosis of a case of malarial disease was based on clinical histories and physical examinations. A case of malarial disease was defined as a person presenting a history of an illness characterized by symptomatology typical of the disease complex known to be caused by plasmodial infections and physical findings typical of malarial pathology. Diagnoses of clinical malaria were made without regard to the blood smear findings.

The chief etiological agent of malarial disease observed in the Clarendon County area as demonstrated by routine monthly thick blood film surveys on the total population was *Plasmodium falciparum*. The cases of malaria were clinically of the simple, nonlocalizing, mild acute type. The symptomatology consisted of irregular, intermittent and remittent night fever, commonly followed by profuse sweating but rarely accompanied by manifest paroxysms. Other chief complaints were frontal and temporoparietal headaches, lumbar backaches, and generalized malaise. Physical findings revealed occasional splenomegaly not greater in size than the number-one type of Boyd's classification of splenomegaly (2) and moderate secondary anemia. All cases were Negroes and the age distributions in both

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§ Director, Division of Preventable Diseases, South Carolina State Board of Health.
[1] The totaquine used in this study was furnished through Defense Supplies Corporation and was manufactured by Merck and Co., Rahway, N. J.

Quantitative Determination of Saccharin*

By HAROLD ROSENBLUM and LEE MILDWORM

A convenient and rapid method for the assay of saccharin and saccharin sodium in tablets is described. The assay is effected by extracting the saccharin with a mixture of chloroform and ether and titrating the extracted saccharin with $N/10$ sodium hydroxide.

ALTHOUGH the U. S. P. XII method for the determination of saccharin in saccharin sodium tablets has been successfully employed, the analysts have always been aware that the method does not isolate the principal ingredient and that the results are dependent on the completeness of the hydrolysis of the NH group to NH_3. Furthermore, in order to insure that this method be workable, a test for ammonia in saccharin, saccharin sodium, and tablets of saccharin sodium is included in the Pharmacopœia. However, no assurance is given that nitrogen containing substances which slowly form ammonia during hydrolysis do not interfere with the assay.

EXPERIMENTAL

In order to circumvent interference from other NH_3 liberating compounds, the proposed method was investigated and found to be reliable and rapid for the determination of saccharin and saccharin sodium in tablet mixtures.

The chemical structure of saccharin is such as to give a strong acid reaction in aqueous solution. Attempts to titrate the solution using phenolphthalein T. S. as indicator gave excellent results. One-half gram of material accurately weighed and dissolved in 75 cc. of water by warming and cooling showed 99.5% saccharin by titration. Furthermore, saccharin was found to be appreciably soluble in a mixture of chloroform and ether (1:1). From these two established facts, the following procedure was evolved for tablet mixtures. Results are given in Table I with a comparison of values obtained with the U. S. P. XII procedure.

Place a sufficient number of tablets equivalent to 1 Gm. of saccharin in a 100-cc. volumetric flask, and add 50 cc. H_2O and 10 cc. N NaOH. Agitate until the tablets have disintegrated. Dilute to volume with water and filter through a dry filter into a dry flask, rejecting the first 20 cc. of filtrate. Transfer 25 cc. of the subsequent filtrate to a separatory funnel, acidify with dilute HCl, and extract the saccharin with 6 x 25-cc. portions of a mixture of chloroform and ether (1:1). Filter the combined extract through a pledget of cotton into a beaker and evaporate to dryness on a steam bath with the aid of a current of air. The residue obtained after evaporation of the solvents is taken up with 75 cc. of boiling water, the solution cooled to room temperature and titrated with $N/10$ NaOH, using phenolphthalein T. S. as indicator.

1 cc. of $0.1N$ NaOH = 0.01832 Gm. saccharin ($C_7H_5O_3NS$)

1 cc. of $0.1N$ NaOH = 0.02413 Gm. saccharin sodium ($C_7H_4O_3NSNa{\cdot}2H_2O$)

DISCUSSION

The interference of stearates in the proposed method was investigated and found to be negligible. In the initial filtration, it was found that practically all of the stearates are removed. Stearic acid if present reacts with sodium hydroxide in the cold too slowly to come through in the filtrate. Tablets containing stearic acid as much as 5 per cent of the weight of the tablet showed little or no stearate interference. The extracted saccharin evaporated to dryness and dried to constant weight at 90° showed approximately a 1 per cent higher assay value than by titration. Furthermore, filtration of the cooled aqueous solution of the extracted saccharin removes all insoluble organic acids that may be present.

TABLE I

Sample	Per Cent Based on Labeled Amount: Proposed Method	U. S. P. XII Method
Saccharin Powder	99.50%	99.4%
Special Granulation (50%)	102.2%	103.6%
Saccharin Tablets, 1/4 gr.	98.8%	99.6%
Saccharin Tablets, 1/2 gr.	97.2%	98.0%
	95.6%	95.6%
Saccharin Tablets, 1 gr.	96.0%	95.7%
Saccharin Sodium Tablets, 1 gr.	103.9%	103.2%

SUMMARY

A rapid, accurate determination of saccharin in saccharin sodium and in tablet mixtures is presented.

* Received June 14, 1946, from Premo Pharmaceutical Laboratories, Inc., New York 13, N. Y.

Evaluation of Totaquine in Treatment of Malaria in an Endemic Malarious Area*,†

By O. W. YEAGER,‡ R. F. REIDER,‡ and G. E. McDANIEL§

A PROGRAM for the purpose of evaluating the usefulness and toxicity of totaquine[1] as an agent in the treatment of naturally occurring malaria was instituted at the Malaria Field Research Station in Manning, S. C. The study which began on June 15, 1945, and extended through November, 1945, was carried on under field conditions in conjunction with other studies on the malarial disease and infections prevalent in the southern portion of Clarendon County, S. C.

Preliminary planning revealed the necessity for recognition of the usual limitations of a drug study carried out under field conditions. Therefore a complete appraisal of all factors involved in drug evaluations was not attempted. The primary objective of this study was simply to ascertain the clinical usefulness and relative toxicity of totaquine as compared with some other established and generally used antimalarial drug with a known degree of toxicity.

Quinine sulfate was chosen as the control drug because it is most frequently used by local physicians throughout the study area in the treatment of malaria, and is the chief ingredient of proprietary antimalarial drugs used by the people in self-medication for the treatment of symptoms alleged to be due to malaria. Quinine sulfate has been used as an antimalarial drug in the area for many years and is most often considered by the people to be the most acceptable drug.

The procedure of study consisted of the treatment at random of cases of clinical malaria with totaquine and quinine sulfate. The schedule of dosage was based on recommendations of the U. S. Army Medical Department (1). The adult therapeutic dose used for both drugs was 1 Gm. orally 3 times daily for two days and then 2 Gm. orally daily for five days. Proportionate dosages were utilized for children, depending upon weight and apparent age. No other drugs were prescribed. Each treated case was followed up to determine the relative acceptability of the two drugs, the occurrence of toxic symptoms, and to make a clinical appraisal of the relative effectiveness of the drugs as therapeutic antimalarial agents. Since this study was operated under natural field conditions, no extensive laboratory investigations were carried out.

The diagnosis of a case of malarial disease was based on clinical histories and physical examinations. A case of malarial disease was defined as a person presenting a history of an illness characterized by symptomatology typical of the disease complex known to be caused by plasmodial infections and physical findings typical of malarial pathology. Diagnoses of clinical malaria were made without regard to the blood smear findings.

The chief etiological agent of malarial disease observed in the Clarendon County area as demonstrated by routine monthly thick blood film surveys on the total population was *Plasmodium falciparum*. The cases of malaria were clinically of the simple, nonlocalizing, mild acute type. The symptomatology consisted of irregular, intermittent and remittent night fever, commonly followed by profuse sweating but rarely accompanied by manifest paroxysms. Other chief complaints were frontal and temporoparietal headaches, lumbar backaches, and generalized malaise. Physical findings revealed occasional splenomegaly not greater in size than the number-one type of Boyd's classification of splenomegaly (2) and moderate secondary anemia. All cases were Negroes and the age distributions in both

* Received July 31, 1946, from the Epidemiology Division, Communicable Disease Center, U. S. Public Health Service, Federal Security Agency, Atlanta, Ga.
† The studies on which this report is based were conducted cooperatively by the South Carolina State Board of Health and the U. S. Public Health Service, Office on Malaria Control in War Areas.
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§ Director, Division of Preventable Diseases, South Carolina State Board of Health.
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the totaquine and quinine groups were uniform and ranged from six months through sixty years of age and over.

In all, 144 cases of malarial disease were treated, 102 with totaquine and 42 with quinine sulfate. Two cases (or 1.9 per cent) of those treated with totaquine and two cases (or 4.7 per cent) of those treated with quinine sulfate reported toxic symptoms of nausea and tinnitus which were severe enough to require discontinuance of treatment. With the exception of the 4 cases which showed moderate untoward reactions, all treated persons reported symptomatic improvement.

In general, totaquine was accepted and taken as readily as quinine sulfate. Both drugs were taken in preference to proprietary antimalarial drugs such as chill tonics.

SUMMARY

During a five-month period a controlled field study of the usefulness and relative toxicity of totaquine (Merck and Co.) as an antimalarial agent was compared with that of quinine sulfate. It was found that both drugs were accepted by the population with equal willingness and in preference to proprietary antimalarials. On the basis of observations in the field, totaquine was found to be about as effective as quinine sulfate in the symptomatic treatment of clinical malarial disease. Totaquine apparently causes no more untoward drug reactions when used under field conditions than quinine sulfate.

More careful evaluation of totaquine is recommended. This evaluation should be carried out where rigid controls can be set up and where necessary laboratory facilities are conveniently available.

REFERENCES

(1) *War Department Technical Bulletin*, TB Med. 72, July 10, 1944.
(2) Boyd, M. F., "Introduction to Malariology," Harvard Univ. Press, Cambridge, Mass., 1930.

The Microscopic Identification of Demerol*

By GEORGE L. KEENAN†

Optical crystallographic data essential for the rapid and accurate microscopic identification of isonipecaine (Demerol) are reported.

DEMEROL is 4-phenyl-piperidine-4-carboxylic acid ethyl ester. It is a white crystalline substance which melts at 30° and is not very soluble in water. Its solutions are very alkaline. The hydrochloride is very soluble in water and in this form the substance is used medicinally. It is said to be a powerful analgesic and may become habit forming like morphine. Legally it is now classified as a narcotic, the name "isonipecaine" having been coined as a suitable designation instead of the trade name Demerol.

The purpose of this paper is to place on record optical crystallographic data which are significant for Demerol hydrochloride thereby affording a rapid and accurate means for its microscopic identification. Data are also given which are characteristic of the crystalline complexes formed by Demerol hydrochloride with the reagents potassium iodide and sodium nitroprusside. (For convenience, unless otherwise qualified, in the following discussion Demerol shall refer to the hydrochloride.)

EXPERIMENTAL

Demerol is a white, crystalline powder, the habit being largely elongated, six-sided prisms. With crossed nicols the extinction is parallel and the sign of elongation positive. Many of the fragments do not extinguish sharply with crossed nicols, these showing one optic axis in the interference figure

* Received July 26, 1946.
† Present address: Strongsville, Ohio, and Baldwin-Wallace College, Berea, Ohio.

when the substance is examined in convergent polarized light (crossed nicols). The significant refraction indices are: $\alpha = 1.545$, $\beta = 1.581$, $\gamma = 1.618$, all ± 0.002. All the indices are readily found on the substance.

Levine in a recent study (1) reported Demerol as giving significant crystalline complexes with several of the common alkaloidal reagents. As a double confirmatory test, the optical crystallographic examination of some of these complexes furnished information that is also useful analytically. This information is especially significant in the case of the crystalline precipitates formed respectively with potassium iodide and sodium nitroprusside.

Potassium iodide reagent (5 Gm. KI in 100 ml. of H_2O) produces fine colorless rods. These can be produced directly on an object slide, the excess reagent removed with small pieces of filter paper, and the precipitate allowed to dry at room temperature. When dry and examined with crossed nicols, the rods show inclined and parallel extinction, and the elongation is positive or negative. Many of the rods do not extinguish sharply with crossed nicols, showing flash biaxial interference figures in convergent polarized light (crossed nicols). The significant refractive indices of this crystalline complex are: $\alpha = 1.605$, $\beta = 1.625$ (most common of the indices), $\gamma = 1.653$, all ± 0.002.

With sodium nitroprusside, Demerol produces long, blade-like plates described and illustrated by Levine (1). He recommends a 5% aqueous solution of sodium nitroprusside as the test reagent. The writer has found that by dropping a small fragment of sodium nitroprusside (about 3 mg.) into the Demerol solution, just as satisfactory a precipitate is obtained. The blades which immediately form are characterized by a shingle-like surface. This characteristic of the surface is considered as quite diagnostic for the substance. After removing the excess liquid, the precipitate can be allowed to dry on the object slide at room temperature and subsequently examined with the polarizing microscope. With crossed nicols, these blades, frequently with obtuse terminations or hexagonal in outline, show inclined extinction and negative elongation. Three refractive indices characterize the substance and are as follows: $\alpha = 1.550$, $\beta = 1.582$, $\gamma = 1.615$, all ± 0.002.

It is also interesting to observe that picrolonic acid reagent (0.5% picrolonic acid in 50% alcohol) on being added to Demerol in a water-drop will at first cause a yellow cloudiness, followed by the gradual formation of spherulites of very fine needles. These aggregates are white and stand out in sharp contrast floating on the yellow liquid, growing to some size and readily observed even with the naked eye. The needles are too slender for microscopic study by the immersion method.

SUMMARY

The optical crystallographic properties of Demerol hydrochloride have been described and also those of two crystalline complexes formed with potassium iodide and sodium nitroprusside, respectively. The data for both of these addition products afford a doubly confirmatory microscopic test for Demerol. Attention is also called to the reaction of Demerol with picrolonic acid reagent.

REFERENCE

(1) Levine, Joseph, *Ind. Eng. Chem., Anal. Ed.*, 16, 408 (1944).

WHO MAKES IT?

The National Registry of Rare Chemicals, Armour Research Foundation, 33rd, Federal and Dearborn Streets, Chicago, Ill., seeks information on sources of supply for the following chemicals:

Thiomorpholine
Chenodesoxycholic acid
Scymnol
Lysidine
Pheophytin
Purpuroxanthin
5,8-Dihydroxy-1,4-naphthaquinone
2,3-Dihydroxyanthraquinone
Alkannin
1-Amino-6-hydroxynaphthalene
o-Aminobenzaldehyde
α,α,-Dichloro-γ-valerolactone
d-Hydroxyglutamic acid
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α,α,-Dichloro-γ-valerolactone
d-Hydroxyglutamic acid
d-Isoleucine
l-Isoleucine
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Pregnanediol, crystalline
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Sodium-*m*-tartrate
Homogentisic acid

Alabama's "Sheep Kill" Laurel, *Kalmia latifolia* L.—A Preliminary Study*

By WILLARD J. HADLEY† and HUGH H. HADEN, JR.‡

"Sheep kill" laurel, or "mountain laurel," a bane to cattle raising in Alabama, was identified as *Kalmia latifolia* L. Half mature leaves were ground and extracted with a series of solvents. Glycosides were present in all extracts prepared with organic solvents. Extractives obtained with isopropyl alcohol were hydrolyzed with hydrochloric acid and aglycone materials separated according to their differences in solubilities. These appeared to be catechol-4-catechuic acid and *cis* and *trans* isomers of *d*-catechol. A hydroxy flavane and a hydroxy chromane were indicated and both appeared to be affected by acid in the same manner as flavones.

SHEEP and cattle have been observed to suffer nausea, vertigo, and progressive paralysis from nibbling the foliage of a laurel which frequents fence rows in Alabama. These animals show little effect from feeding on the young plant, and deer and goats appear little affected on eating the laurel at any stage of its development. Honey that is made by bees which have access to laurel, rhododendron, and others of the Heath family is regarded as unsafe for human food, but the bees store and consume it with safety. Household decoctions of laurel leaves have been given in diarrhea and employed as lotions in skin diseases. Preparations of the leaves have even acquired reputations, in some sections, as palliatives in syphilis.

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In this first crop, taken before the laurel had stored much of its toxic constituents, we have found minute amounts of 2 glycosides. The aglycone portions of these appear to be protocatechuic acid and *d*-catechol (*cis* and *trans* isomers), known compounds (3–5), and the sugars, fructose, glucose, xylose, and arabinose. Chemical confirmation is incomplete. Photomicrographs of the aglycone materials were obtained. No report has been found in the literature of pharmacologic testing of these compounds. We have thus far been unable to isolate amounts adequate for thorough chemical study or for physiological examination, and will resume the investigation

material were reported variously. The present study was undertaken for the purpose of isolation and identification of "andromedotoxin" or whatever poisonous agent is contained in Alabama's "Sheep Kill" laurel. Future work will include observation of the physiological properties of the principles we have found indicated in this "mountain laurel."

EXPERIMENTAL

Air-dried leaves of the laurel were ground to number 20 powder.

The "drug constants" of the United States Pharmacopœia were found. The averages of these were as follows:

"Characteristic" or "Constant"	%
Moisture	6.70
Total ash	3 43
Acid-insoluble ash[1]	0.20
Ligroin-soluble extractives	4.54
Ether-soluble extractives	10.30
Alcohol-soluble extractives	30.80
Hydroalcohol-soluble extractives	5.67
Water-soluble extractives	17.25

The drug was extracted, in Soxhlet apparatus, with 5 solvents, successively employed; viz., ligroin, ether, chloroform, alcohol, water. None of these extracts gave indications of the presence of alkaloids with the usual precipitants.

From the ligroin extract a small amount of paraffin-type wax was obtained, by chilling an 80% alcohol mixture. It melted indefinitely, and did not yield to fractionation by distillation *in vacuo*. The ligroin extract was observed to contain, also, some fixed oil, which was associated with resins that were made up of high molecular weight acids. The saponification number of the oily mixture was 126 (8). Sterol tests (9, 10) were faint. Glycoside tests (11) were clear but weak.

The ether extract yielded to cold water some catechol-type tannins (green in ferric chloride solution), some glycosidic material (11), and sugars. These latter formed addition products with 2,4-dinitrophenylhydrazine but were incapable of restoring color to Schiff's reagent. This was evidence that the sugars were ketones.

The chloroform extract gave tests which paralleled those of the ether extract. This extract also bore difficultly soluble, fat-like material, indifferent toward all reagents and judged to be resenes.

The alcohol extract gave the indications of glycosides, of catechol tannins and of ketonic sugars above, and yielded extractive material which was insoluble in alkali bicarbonate but soluble in alkali hydroxide, confirming phenols. An osazone formed in two minutes, the reported time for fructosazone (12).

Molisch tests (11) and Fehling's tests (13) were both positive on an acetone extract of a fresh sample, but the Molisch tests were negative on the same extract after the extract had been warmed with 5% HCl. These observations lent evidence that the glycosides contained in the drug were easily hydrolyzable.

The water extract, fifth in the series, above, gave Molisch tests and catechol tannin tests and yielded an osazone in five to fifteen minutes (clearly a mixture). The time factor, here, suggested glucose, xylose, and arabinose sugars (12). These are all aldehydes, and tests for carbonyl compounds in the ether extract, above, and the time of formation of the osazone from alcohol extractives, above, indicated ketonic sugars, specifically fructose. But glucose, xylose, or arabinose could conceivably be only a portion of the carbohydrate part of a glycoside, between the aglycone and a fructose end unit. The carbohydrate would be cleaved more easily than the aglycone would be liberated, and, thus, while free fructose would be expected in the extracts obtained with organic solvents, above, extraction of the drug with water would be accompanied by liberation of the aglycone from the immediately adjacent portion of the carbohydrate. This would give in the water extract these aldehydic sugars, which were not indicated in the ether, chloroform, and alcohol extracts. No characteristic physical form could be observed microscopically in the mixture of osazones.

Isopropyl Alcohol Extract.—One kilogram of drug was extracted thoroughly, in Soxhlet apparatus, with isopropyl alcohol. The extract was acidified with HCl, then evaporated at moderate heat almost to dryness, and, finally, stirred into 5 L. of cold water. A flocculent, grayish precipitate, consisting perhaps of aglycone substances and secondary glycosides, was recovered by centrifuging and washed with an ether-acetic acid mixture. These washings yielded small amounts of two kinds of crystals, in a viscous, amber-colored matrix, the one filamentous and lacy and the other in elongated needles (10×). The mixture was triturated with sand and extracted with chloroform. The chloroform extract yielded a few of the needle-shaped crystals but none of the other crystals. The amount obtained was too small to permit recrystallization. The crude material melted at 190–210°, with decomposition. It produced a green coloration with ferric chloride, then red with sodium bicarbonate. These tests, together with the melting temperature observed, suggested catechol-4-carboxylic acid, called also protocatechuic acid and veratric acid (3). A photograph of these crystals, in their dark, viscous matrix, is given in Fig. 1 (*A*) (100×).

When a mixture of the two kinds of crystalline material was extracted with, first, cold glacial acetic acid and, second, chloroform, the former of these extracts was found to contain a small amount of the lacy crystalline material, above, and the latter extract yielded a few of the needle-shaped crystals.

[1] Found to contain calcium carbonate and ferrous sulfide.

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At the same time that the effects of this plant came to our attention, botanists at Tuskegee Institute and the George Washington Carver Foundation began a systematic identification of this bane to cattle growers. These investigators established it as *Kalmia latifolia* L., popularly called "mountain laurel," "broad-leaf laurel," "calico bush," and "spoonwood" (1, 2). They volunteered to supply us with quantities sufficient for preliminary chemical examination, and collected for this study about 10 pounds of the leaves in Marion County, Ala., in the month of June, a time when the plant is of nearly normal size but scarcely mature.

In this first crop, taken before the laurel had stored much of its toxic constituents, we have found minute amounts of 2 glycosides. The aglycone portions of these appear to be protocatechuic acid and *d*-catechol (*cis* and *trans* isomers), known compounds (3–5), and the sugars, fructose, glucose, xylose, and arabinose. Chemical confirmation is incomplete. Photomicrographs of the aglycone materials were obtained. No report has been found in the literature of pharmacologic testing of these compounds. We have thus far been unable to isolate amounts adequate for thorough chemical study or for physiological examination, and will resume the investigation when older laurel is available. Director R. W. Brown, of the George Washington Carver Foundation, and Professor H. J. Romm, of Tuskegee Institute, are collecting for us larger quantities of the leaves, taken at a more advanced stage of the plant's growth. The extractive principles so far obtained have appeared easily altered chemically. We shall, in repeating certain parts of this work, make some significant adaptations in procedure in the hope of overcoming losses at present sustained.

A toxic extractive was obtained from *Kalmia latifolia* by Lasché, in 1889, and named by him "andromedotoxin" (6). Lasché and DeZaayer found "andromedotoxin" nonnitrogenous. Their reported findings suggest that it was a glycoside (7). It is evident that "andromedotoxin" was a mixture. The melting points of their

* Received July 25, 1946, from the Pharmacy Department, Division of Pharmaceutical Chemistry, Howard College, Birmingham, Ala.
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material were reported variously. The present study was undertaken for the purpose of isolation and identification of "andromedotoxin" or whatever poisonous agent is contained in Alabama's "Sheep Kill" laurel. Future work will include observation of the physiological properties of the principles we have found indicated in this "mountain laurel."

EXPERIMENTAL

Air-dried leaves of the laurel were ground to number 20 powder.

The "drug constants" of the United States Pharmacopœia were found. The averages of these were as follows:

"Characteristic" or "Constant"	%
Moisture	6.70
Total ash	3.43
Acid-insoluble ash[1]	0.20
Ligroin-soluble extractives	4.54
Ether-soluble extractives	10.30
Alcohol-soluble extractives	30.80
Hydroalcohol-soluble extractives	5.67
Water-soluble extractives	17.25

The drug was extracted, in Soxhlet apparatus, with 5 solvents, successively employed; viz., ligroin, ether, chloroform, alcohol, water. None of these extracts gave indications of the presence of alkaloids with the usual precipitants.

From the ligroin extract a small amount of paraffin-type wax was obtained, by chilling an 80% alcohol mixture. It melted indefinitely, and did not yield to fractionation by distillation *in vacuo*. The ligroin extract was observed to contain, also, some fixed oil, which was associated with resins that were made up of high molecular weight acids. The saponification number of the oily mixture was 126 (8). Sterol tests (9, 10) were faint. Glycoside tests (11) were clear but weak.

The ether extract yielded to cold water some catechol-type tannins (green in ferric chloride solution), some glycosidic material (11), and sugars. These latter formed addition products with 2,4-dinitrophenylhydrazine but were incapable of restoring color to Schiff's reagent. This was evidence that the sugars were ketones.

The chloroform extract gave tests which paralleled those of the ether extract. This extract also bore difficultly soluble, fat-like material, indifferent toward all reagents and judged to be resenes.

The alcohol extract gave the indications of glycosides, of catechol tannins and of ketonic sugars above, and yielded extractive material which was insoluble in alkali bicarbonate but soluble in alkali hydroxide, confirming phenols. An osazone formed in two minutes, the reported time for fructosazone (12).

Molisch tests (11) and Fehling's tests (13) were both positive on an acetone extract of a fresh sample, but the Molisch tests were negative on the same extract after the extract had been warmed with 5% HCl. These observations lent evidence that the glycosides contained in the drug were easily hydrolyzable.

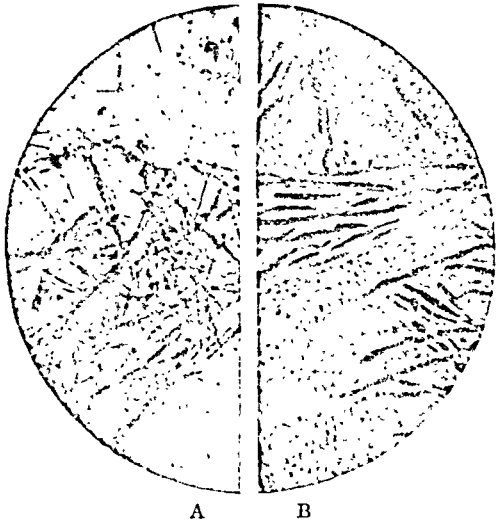
The water extract, fifth in the series, above, gave Molisch tests and catechol tannin tests and yielded an osazone in five to fifteen minutes (clearly a mixture). The time factor, here, suggested glucose, xylose, and arabinose sugars (12). These are all aldehydes, and tests for carbonyl compounds in the ether extract, above, and the time of formation of the osazone from alcohol extractives, above, indicated ketonic sugars, specifically fructose. But glucose, xylose, or arabinose could conceivably be only a portion of the carbohydrate part of a glycoside, between the aglycone and a fructose end unit. The carbohydrate would be cleaved more easily than the aglycone would be liberated, and, thus, while free fructose would be expected in the extracts obtained with organic solvents, above, extraction of the drug with water would be accompanied by liberation of the aglycone from the immediately adjacent portion of the carbohydrate. This would give in the water extract these aldehydic sugars, which were not indicated in the ether, chloroform, and alcohol extracts. No characteristic physical form could be observed microscopically in the mixture of osazones.

Isopropyl Alcohol Extract.—One kilogram of drug was extracted thoroughly, in Soxhlet apparatus, with isopropyl alcohol. The extract was acidified with HCl, then evaporated at moderate heat almost to dryness, and, finally, stirred into 5 L. of cold water. A flocculent, grayish precipitate, consisting perhaps of aglycone substances and secondary glycosides, was recovered by centrifuging and washed with an ether-acetic acid mixture. These washings yielded small amounts of two kinds of crystals, in a viscous, amber-colored matrix, the one filamentous and lacy and the other in elongated needles (10×). The mixture was triturated with sand and extracted with chloroform. The chloroform extract yielded a few of the needle-shaped crystals but none of the other crystals. The amount obtained was too small to permit recrystallization. The crude material melted at 190–210°, with decomposition. It produced a green coloration with ferric chloride, then red with sodium bicarbonate. These tests, together with the melting temperature observed, suggested catechol-4-carboxylic acid, called also protocatechuic acid and veratric acid (3). A photograph of these crystals, in their dark, viscous matrix, is given in Fig. 1 (*A*) (100×).

When a mixture of the two kinds of crystalline material was extracted with, first, cold glacial acetic acid and, second, chloroform, the former of these extracts was found to contain a small amount of the lacy crystalline material, above, and the latter extract yielded a few of the needle-shaped crystals.

[1] Found to contain calcium carbonate and ferrous sulfide.

The former deposit was laboriously separated with a teasing needle, under a magnifying glass, into what appeared as main threads and what appeared as filler threads. These "main threads" melted, without recrystallization, at 177–180°. The "filler threads" melted, without recrystallization, at 240–245°. Both produced a green coloration with ferric chloride. Both were found soluble in water, alcohol, ether, and dilute alkali hydroxide, but not in dilute alkali bicarbonate. These tests, together with the melting temperatures observed, suggested the two geometric isomers of *d*-catechol (4, 5). A photograph of this crystalline material, in its viscous, amber-colored matrix, is given in Figure 1 (*B*) (100×).



A B

FIGURE 1

The last of the drug on hand, somewhat less than 1 Kg., was percolated with isopropyl alcohol. The percolate was caught and distributed in a mixture of purified sand and gravel. The solvent was removed by distillation, and the residue was steam distilled. The distillate contained volatile oil and a small amount of crystalline material which satisfied the tests for catechol-4-carboxylic acid. This material was rapidly discolored by exposure to air, precluding recovery. The residue of steam distillation was extracted with glacial acetic acid. This extract was distributed on sand and gravel and evaporated to dryness and the absence of acetic acid odor. The residue was then treated with boiling water. The water solution, dark brown, gave tests for the presence of catechol-4-carboxylic acid, but yielded no crystalline substance. Instead, fairly abundant brown-black, amorphous material settled out. It was apparent that the hot acid produced changes in the flavanol and the chromanol which gave deeply colored, amorphous compounds. The acid could be expected to produce ring-opening in either of these, giving reduced chalcones that gave cations which conferred color (14).

SUMMARY

Alabama's "sheep kill" laurel, which was identified by collaborators as *Kalmia latifolia* L., was found to store two glycosides, as it approached maturity, but gave no indications of alkaloids. The writers hypothesize that a mixture of these glycosides was Lasché and DeZaayer's "andromedotoxin." Small quantities of aglycone materials were obtained in impure state. The preliminary chemical study and the photomicrographs obtained suggested catechol-4-carboxylic acid and both the geometric isomers of *d*-catechol. Glucose, xylose, arabinose, and fructose were the indicated sugars.

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The Thiamine Content of Herbs and Medicinal Plants*

By ALEXANDER S. CHAIKELIS†

By means of a photofluorometric assay technique, eliminating the elution phase, devised to investigate the thiamine content of plant material ranging from rhizomes and roots to barks, leaves, and flower tops, the thiamine concentration in herbs and medicinal plants was determined. A spot check analysis of 41 genera belonging to 20 distinct and separate botanical families showed a thiamine range from 125 to 2880 micrograms per 100 Gm. of dried substance. This vitamin B_1 content was postulated as a possible modus operandi of the many infusions and decoctions of medicinal herbs prescribed in cases of anorexia, dyspepsia, general debility, and nervousness.

FOR MANY CENTURIES the use of plants in the alleviation and cure of bodily ills received wide attention but little official sanction until in the reign of King Henry VIII Parliament enacted what is now popularly known in England, "The Herbalists' Charter" (1).

Healers, like Nicholas Culpepper (1616–1654) in England and Samuel Thompson (1769–1843) in America, did much to advance the study and practice of herbalism. Apart from the claims made by the herbalists, the use of herbs as sources of drugs, like those described in articles and books on pharmacognosy and/or materia medica, has done much to justify their usefulness in medicine (2–7).

With our present knowledge of the various sources of vitamins, can it be that the cumulative beneficial results produced by the use of herbs, exclusive of their medicinal properties, might be the effects of increased vitamin intake? To obtain information along this line of thought, it was decided to make a spot check on some herbs and other plants, emphasis to be on those which were and are, at present, described as possessing tonic, stomachic, and stimulant actions in cases of dyspepsia, constipation, general debility, and nervousness. Since all higher plants are able to not only synthesize thiamine but to excrete it through the roots, it seemed logical to investigate the existence and quantity of thiamine in these and other parts of the dried plants that had recognized medicinal value.

Samples of the herbs and medicinal plants were purchased in the dried state from a reliable distributor of such material. The plants so bought represented 41 genera belonging to 20 separate botanical families. This number of specimens was considered to be an adequate sampling of the entire group of medicinals.

EXPERIMENTAL

Based on the idea of procedure for thiochrome determination described by Hennessy (8) and the digestion method of plant material to release the thiamine in the free state as well as the phosphorylated form (cocarboxylase) suggested by Platt and Block (9), the thiamine content of the plant material was analyzed by the following method devised to fit the demands of the material under investigation and the requirement of a quick, reliable technique.

A. Reagents Required.—The many reagents needed to effectively carry out the thiochrome determinations included those listed in the U. S. P., those enumerated by Platt and Block (9), and those, such as the needed *standard solutions*, described in (10).

The digesting enzymes papain and clarase before use were treated in the following manner: A 5-Gm. batch of each enzyme is suspended in 50 cc. distilled water and shaken thoroughly. To this suspension is added 50 cc. isobutanol, and the mixture again shaken in a separatory funnel. Discard the supernatant isobutanol fraction, repeating the process until the isobutanol portion when oxidized with NaOH and $K_3Fe(CN)_6$ shows no evidence of thiochrome when examined in a photofluorometer.

The aqueous fraction is then treated with ethanol to precipitate the enzymes, filtered, and the residue dried *in vacuo*. The dry amorphous powder is then ready for use in the digestion phase of the treatment section of the procedure.

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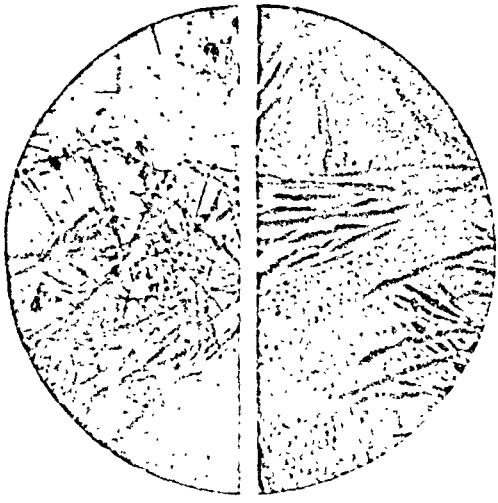

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TABLE I—COMPOSITE SUMMARY OF THIAMINE CONTENT IN VARIOUS DRIED HERBS AND OTHER MEDICINAL PLANTS

Common Name	Genus and Species	Family	Parts Used	Av Dose in Gm.	Thiamine in γ/Gm	Thiamine in γ/Dose	Thiamine in γ/100 Gm.	Action and Method of Administration
Agrimony	*Agrimony eupatorium* Linn.	Rosaceae	Entire herb	1	2.355	9.42	235.5	Tonic, as an infusion: 1 oz. to 1 pt. water
Angostura	*Galipea officinalis*	Rutaceae	Bark	2	11.286	22.56	1128.6	Bitter tonic, as above, 2 fl. oz. t. i. d.
Balmony	*Chelone glabra* Linn.	Scrophulariaceae	Leaves	1	6.594	26.37	659.4	Tonic, for constipation, as above, 2 fl. oz. t. i. d.
Barberry	*Berberis Aquifolium* (Pursh.)	Berberidaceae	Root bark	2	10.038	20.07	1003.8	Bitter tonic, powdered bark, 20 gr. t. i. d.
Bay Laurel	*Laurus nobilis* Linn.	Lauraceae	Leaves		7.928		792.8	Flavoring: spice for meats, poultry, meat sauces
Black Alder	*Ilex verticillata* Linn.	Aquifoliaceae	Bark	2	22.461	44.94	2246.4	Tonic, as infusion, 2 fl. oz. q. i. d.
Blessed Thistle	*Cnicus benedictus* (Goetn.)	Compositae	Entire herb	4	5.879	23.52	587.9	Tonic, as above
Boneset	*Eupatorium perfoliatum* Linn.	Compositae	Leaves	2	5.378	10.74	537.8	Tonic; as above, 2 fl. oz. q. i. d. Served cold
Buchu	*Barosma betulina* Bartling and Wendl.	Rutaceae	Leaves	2	19.656	39.30	1965.6	Stimulant, as above, 2 fl. oz. q. i. d.
Bugleweed	*Lycopus uniflorus* Linn.	Labiatae	Entire herb	4	2.960	11.85	296.0	Calmative; as above
Calamus	*Acorus Calamus* Linn.	Araceae	Rhizome and roots	3	5.338	16.02	533.8	Aromatic bitter; as above
Caraway	*Carum Carvi* Linn.	Umbelliferae	Seeds	1	7.222	7.23	722.2	Stimulant, carminative; 2 fl. oz. t. i. d. as an infusion
Cascarilla	*Croton Eluteria* Bennett	Euphorbiaceae	Bark	2	8.763	17.52	876.3	Bitter; as infusion, a 2-fl. oz. dose t. i. d.
Catnip	*Nepeta cataria* Linn.	Labiatae	Leaves	4	5.600	22.38	560.0	Tonic; as infusion, 2 fl. oz. q. i. d.
Celery	*Apium graveolens* Linn.	Umbelliferae	Seeds	2	7.928	15.81	792.8	Stimulant, as infusion, 2 fl. oz. t. i. d.
Chamomile	*Matricaria Chamomilla* Linn.	Compositae	Flower tops	15	11.542	173.13	1154.2	Bitter, as infusion, 2 fl. oz. q. i. d.
Cherry Tree	*Prunus serotina* Linn.	Rosaceae	Leaves	2	18.720	37.38	1872.0	Bitter tonic; as infusion, 2 fl. oz. t. i. d.
Condurango	*Marsdenia Condurango* Reichenbach f.	Asclepiadaceae	Bark	4	10.340	41.25	1034.0	Bitter; as infusion, as above
Dandelion	*Taraxacum officinale* Weber	Compositae	Rhizome and roots	4	15.774	63.09	1577.4	Simple bitter; as infusion, 2 fl. oz. q. i. d.
Dogwood (Jamaica)	*Piscidia erythrina* (Jacques)	Leguminosae	Root bark	2	5.652	11.31	565.2	Bitter tonic; as infusion, 2 fl. oz. t. i. d.
Feverfew	*Chrysanthemum Parthenium* Bernh.	Compositae	Entire herb	2	5.024	10.05	502.4	Calmative in hysteria; as infusion, 2 fl. oz. t. i. d.
Garden Sage	*Salvia officinalis* Linn.	Labiatae	Leaves	4	8.544	34.17	854.4	Carminative; as infusion for dyspepsia, as above
Gentian	*Gentiana lutea* Linn.	Gentianaceae	Rhizome and roots	1	4.774	4.77	477.4	Simple bitter; as infusion, 2 fl. oz. q. i. d.
Ghost Root	*Angelica archangelica* Linn.	Umbelliferae	Rhizome and roots	2	6.832	13.68	683.2	Bitter; as above
Ground Ivy	*Nepeta hederacea* Linn.	Labiatae	Entire herb	4	6.029	24.12	602.9	Tonic; as infusion in dyspepsia, as above
Horseradish	*Cochlearia armoracia* Linn.	Cruciferae	Rhizome and roots	4	5.024	20.10	502.4	Stomachic; as infusion, 2 fl. oz. t. i. d.
Moonseed	*Menispermum canadense* Linn.	Menispermaceae	Rhizome and roots	1	8.653	8.64	865.3	Simple bitter; as above, 2 fl. oz. t. i. d.
Musk Root	*Ferula Sumbul* Hooker filius	Umbelliferae	Rhizome and roots	2	6.537	13.08	653.7	Calmative; as an extract in 8-gr. doses
Parsley	*Carum petroselinum* (Benth.)	Umbelliferae	Leaves	..	4.396	....	439.6	Flavoring; used ad libitum
Parsley	*Carum petroselinum* (Benth.)	Umbelliferae	Rhizome and roots	3	1.256	3.78	125.6	Calmative; as infusion, 2 fl. oz. t. i. d.
Prickly Ash	*Xanthoxylum americanum* Miller	Rutaceae	Bark	2	28.800	57.60	2880.0	Stimulant; as infusion, 2 fl. oz. q. i. d.
Quassia	*Picrasma excelsa* Panchon	Simarubaceae	Heartwood	1/2	18.386	9.18	1838.6	Bitter tonic; 0.5 cc. as fluidextract
Scullcap	*Scutellaria lateriflora* Linn.	Labiatae	Entire herb	1	6.908	6.90	690.8	Tonic; as infusion, 4 fl. oz. q. i.d.
Snakeroot (Can.)	*Asarum canadense* Linn.	Aristolochiaceae	Rhizome and roots	2	4.384	8.76	438.4	Aromatic bitter; as infusion, 2 fl. oz. t. i. d.
Snakeroot (Va.)	*Aristolochia serpentaria* Linn.	Aristolochiaceae	Rhizome and roots	1	3.657	3.66	365.7	Aromatic bitter; as infusion, as above
Southernwood	*Artemisia abrotanum* Linn.	Compositae	Entire herb	4	2.000	8.00	200.0	Emmenagogue; as infusion, 2 fl. oz. q. i. d.
Strawberry (wild)	*Fragaria virginiana* Linn.	Rosaceae	Leaves	4	2.800	11.19	280.0	Bitter tonic; as infusion, 2 fl. oz. t. i. d.
Tansy	*Tanacetum vulgare* Linn.	Compositae	Entire herb	2	5.338	10.68	533.8	Stimulant, antispasmodic; as infusion, as above
Thyme (common)	*Thymus vulgaris* Linn.	Labiatae	Leaves	4	5.024	20.10	502.4	Stimulant, antispasmodic; as above
White Ash	*Fraxinus americana* Linn.	Oleaceae	Bark	2	20.585	41.16	2058.5	Bitter tonic; as infusion, 2 fl. oz. t. i. d.
White Cinnamon	*Cinnamomum Cassia* (Blume)	Lauraceae	Bark	2	4.270	8.54	427.0	Aromatic bitter; as above
Yellow Dock	*Rumex crispus* Linn.	Aristolochiaceae	Rhizome and roots	4	10.261	41.04	1026.1	Stomachic, tonic; as above

B. Treatment of the Substance to Be Analyzed.—1. Place 10 Gm. of the substance, dried and finely ground, in a 250-cc. flask.

2. Add 90 cc. $N/10$ H_2SO_4, mix thoroughly, and then add 0.2 Gm.-batches each of the purified papain and clarase.

3. Now add 10 cc. of the $M/10$ sodium acetate-acetic acid buffer solution. Mix well (check *p*H with bromcresol green so that it is 4.0; correct with either 10% HCl or 10% NaOH).

4. Stopper flask and heat the mixture at 40–45° for from sixteen to eighteen hours.

5. Remove flask, add 0.2 cc. glacial acetic acid, and heat mixture to 80° to coagulate the proteins.

6. Cool flask to room temperature; centrifuge a 20-cc. portion, and save the clear supernatant aqueous layer for the analysis.

7. Oxidation (by means of alkaline ferricyanide) of thiamine to thiochrome, which is extracted by isobutanol, is followed by measurement of the fluorescence in ultraviolet light of the thiochrome formed. The thiamine pyrophosphate also forms a fluorescent thiochrome, which is not, however, soluble in isobutanol—hence one reason for the enzyme treatment to hydrolyze the thiamine pyrophosphate to thiamine.

8. With a Coleman Universal Spectrophotometer #11 adapted with an ultraviolet illuminator and the requisite filters for thiamine study, determine the degree of fluorescence using the substitute working standard of quinine sulfate (3 gamma per cc.). See details in the direction booklet (11).

RESULTS

The values for the vitamin B_1 content of the 41 genera of herbaceous medicinal plants are represented in Table I. The thiamine, in micrograms per gram of dried substance, as listed in the table represents the *mean* value of three distinct and separate samples of each plant studied.

Of the total number of plants studied in this spot check, it can be seen by reference to Table I that a group of seven medicinal plants contain thiamine in excess of 1500 γ/100 Gm. of substance. These seven plants arranged in order of the highest thiamine content are: Prickly Ash (*Xanthoxylum americanum* Miller), Black Alder (*Ilex verticillata* Linn.), White Ash (*Fraxinus americana* Linn.), Buchu (*Barosma betulina* Bartling & Wendland), Cherry (*Prunus serotina* Linn.), Quassia (*Picrasma excelsa* Panchon), and Dandelion (*Taraxacum officinale* Weber).

On the other hand, five genera show a thiamine content of less than 300 γ/100 Gm. of dried substance. Arranged in order from the lowest to the highest concentration these five herbs are: Parsley (root) (*Petroselinum sativum* Hoffman), Southernwood (*Artemisia abrotanum* Linn.), Agrimony (*Agrimony eupatorium* Linn.), Strawberry (wild) (*Fragaria virginiana* Linn.), and Bugleweed (*Lycopus uniflorus* Linn.). The remaining herbs in the series range from 365–1154 γ/100 Gm. of substance.

DISCUSSION

Many factors influence the effectiveness with which a plant synthesizes vitamins. Therefore the values reported in this paper cannot be considered as constant but merely as indicative of the concentration of thiamine contrary to the claim made that leaves, for instance, belonging to higher plants regardless of the botanical family source contain a constant thiamine concentration equal to 75 micrograms per 100 Gm.

It is an accepted fact that animals (including man) require a daily intake of thiamine because this vitamin is not stored in the organism although, as Westerbrink (12) reported, relatively higher concentrations are found in the liver, kidneys, heart, muscle, and brain than in the blood stream. The animal organism absorbs only as much thiamine as is needed for the time being, all excess is excreted and to a small extent destroyed in the body.

The physiological action of thiamine in the animal organism in the form of its pyrophosphoric acid ester is intimately concerned with carbohydrate, fat, and water metabolism. Thus, glycogen, the specific animal carbohydrate, requires the presence of this vitamin to be properly metabolized. The fat metabolism is influenced by vitamin B_1 only in so far as the synthesis of fat from carbohydrate is concerned. The water metabolism in the animal organism seems connected with the action of this vitamin, probably through the carbohydrate metabolism. Edema and water imbibitions in the heart and in other organs are symptoms of thiamine inadequacy. Vitamin B_1 deficiency affects, first of all, the emotions and the tonus of the nervous system, for this substance takes part in the conduction of nerve impulses as shown by Minz (13) that acetylcholine and vitamin B_1 are liberated during impulse conduction.

Further symptoms of thiamine deficiency are loss of appetite (anorexia), lowered physical endurance associated with unusual susceptibility to fatigue, gastrointestinal

disturbances (dyspepsia, constipation), muscular weakness, and a decrease of the blood pressure.

Attention is called to the fact that it is precisely for such discomforts that many herb infusions and decoctions, known as "tonics," have been prescribed. The indications are that these prescriptions materially benefited the ailing individual, at least in a small measure, by providing him with readily absorbable thiamine and its phosphorylated form at a time when his system was in great need of these items.

SUMMARY

1. The thiamine content of a spot check analysis of 41 genera of herbs and medicinal plants was determined by means of the photofluorometric technique.

2. A photofluorometric assay technique was devised employing the suggestions of Hennessy (8) and those of Platt and Block (9).

3. These 41 genera of herbs and medicinal plants belonging to 20 separate botanical families showed a thiamine content ranging from 125–2880 micrograms per 100 Gm. of dried substance.

4. The rational basis for the use of herb infusions and decoctions in the form of "tonics" is suggested on the basis of the physiological action of thiamine in the animal organism.

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Persistence of Penicillin in Saliva After Local Oral Application*

By MILTON GJELHAUG LEVINE and ROBERT E. HOYT

Penicillin for local application to the mouth area was administered in a chicle base chewing gum, in flavored paraffin, and in a mouthwash. The retention time of penicillin in the saliva for each of these dosage forms with varying concentrations of the drug is recorded in this report.

THE USE of penicillin in the buccal cavity has been suggested in hemolytic streptococcus infections, tonsillitis, Vincent's disease (acute ulcerative gingivostomatitis), Ludwig's angina, for lesions of the oral cavity involving loss of surface, and for prophylactic use in operative procedures of the mouth. Of all these, the most convincing therapeutic evidence has been adduced for the use of local preparations in Vincent's angina.

Our own experiences and those reported by other investigators (1, 2, 9, 10) indicate that in most cases Vincent's disease may be cured in from twenty-four to forty-eight hours by the local application of penicillin. The results for other infections so far have been inconclusive, although preliminary reports (2–5) indicate that Group A streptococci may be reduced in number and even eliminated by local applications of penicillin. However, more satisfactory results may be obtained by parenteral administration of the drug. Parenteral administration of penicillin has also proved to be of value in Ludwig's angina (6, 7).

The treatment of acute ulcerative stoma-

* Received Aug. 12, 1946, from the Institute of Experimental Medicine, College of Medical Evangelists, Los Angeles, Calif.

titis by local application of penicillin is obviously preferable to parenteral administration, since most cases are ambulatory, seen either by the physician or dentist. Two preparations have been suggested for this purpose: the pastille (2), and the mouthwash (1). Gregory and Lang (2) incorporated 500 units of penicillin into a pastille. Penicillin was said to be present in the saliva up to thirty minutes after removal of the pastille although no unitage was mentioned. The mouthwash suggested by Schmidt and Horwitz (1) contained 200 units of penicillin per cubic centimeter of normal saline. The mouth was washed by swishing one mouthful of the solution around the mouth once every four hours. Again, no figures were reported on persistence of the drug in the saliva. Other preparations have been suggested: Strock (8) has reported having patients hold a solution containing 5000 units per cubic centimeter in the mouth for periods up to one hour. Denny and co-workers (9) swabbed a solution containing 250 units per cubic centimeter on the Vincent's ulcers four times daily. In both instances, the methods were cumbersome and the assay data supplied were inadequate.

To supply more complete data on the persistence of penicillin in the mouth after local application the following experimental study was undertaken.

EXPERIMENTAL

For local application to the mouth area, penicillin was administered in chewing gum, in a mouthwash, and in a flavored paraffin base. The gum contained 400 units of penicillin per stick (1 Gm. of chicle base flavored with saccharin and wrapped in lead foil). The gum was chewed during the entire period of observation. The mouthwash was prepared by combining penicillin with dibasic and monobasic sodium phosphate to give a *p*H of 6. This tablet was dissolved in 20 cc. of water; the whole volume was then swished around in the mouth for one minute and expectorated.

The third medication consisted of calcium penicillin in flavored paraffin and was chewed during the entire observation period.

Assays of penicillin in saliva (made by the Oxford cup technique) were employed as an index of persistence of the drug in the mouth. The results are reported in Table I. The sign > indicates greater than, the sign < indicates less than the number of units reported. Each figure represents an average of at least four observations on four different subjects.

TABLE I.—PERSISTENCY OF PENICILLIN IN SALIVA IN UNITS PER CC. OF SALIVA

Time, Hr.	Gum 400 Units Total	Paraffin, 10,000 Units Total	Paraffin, 20,000 Units Total	Paraffin, 40,000 Units Total	Mouthwash[a] 200 Units/Cc.	Mouthwash[a] 5000 Units/Cc.	Mouthwash[a] 10,000 Units/Cc.
1/2	>1	>1	>1	>1	0.5	>1	>1
1	>1	>1	>1	>1	0.25	>1	>1
1 1/2	1	>1	>1	>1	<0.25	>1	>1
2	0.5	1	>1	>1	<0.25	>1	>1
2 1/2	0.25	1	>1	>1	0	>1	>1
3	<0.25	1	>1	>1	....	>1	>1
3 1/2	....	<0.25	0.5	>1	....	0.25	0.5
4	....	0	0.5	>1	....	<0.25	0.5

[a] A total of 20 cc. of mouthwash was used in each case.

DISCUSSION

The persistence of penicillin in the mouth for periods up to four hours in length depends on the amount of penicillin involved regardless of the preparation. Of special interest is the prolonged presence in the mouth of penicillin after washing with the medicated solution for only one minute. The choice of an oral preparation will depend on the particular circumstance. Where the patient can chew for long periods, the gum or paraffin type is preferable, since the total number of units required for a continuous high level is less than with the mouthwash. However, where the patient cannot chew the mouthwash is desirable.

It must be emphasized that if a mouthwash is used, no food or liquid should be employed since these will undoubtedly dilute the penicillin remaining in the mouth.

It is difficult to set a therapeutic level for penicillin in the mouth since no chemotherapeutic investigation has been carried out on the controversial organism thought to be the cause of Vincent's disease. However, since 0.3 of a unit of penicillin (10) is the upper limit reported to be needed for bacteriostasis of susceptible bacteria in plasma, such a figure must arbitrarily be used in interpreting the data herein presented for saliva.

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The Interaction of Sympathomimetic Pressor Amines: Inversion of the Vonedrine Pressor Action by Paredrine*

By RAYMOND P. AHLQUIST†

The presence of Paredrine [β(*p*-hydroxyphenyl)-isopropylmethylamine] in the anesthetized dog causes the pressor action of Vonedrine (β-phenyl-*n*-propylmethylamine) to become inverted to a depressor action. Correlation of the arterial pressure and the rate of blood flow in the hind leg or head shows that this depressor action is due to peripheral vasodilation. Repeated doses of Vonedrine alone produce a diminishing pressor response but no depressor action. Other amines, including epinephrine, ephedrine, tyramine, Sympatol, and Propadrine, do not invert Vonedrine and are themselves not inverted by Paredrine. Benzylimidazoline, a sympathomimetic antipressor agent, does not invert Vonedrine. The findings in this study indicate that the inversion of the Vonedrine pressor response by Paredrine may be an example of true reversal of action and not an unmasking of a normally present response.

THE CARDIOVASCULAR response to a sympathomimetic pressor amine is always modified by the presence of a previous dose of the same or another amine. The pressor response may be augmented or diminished or converted completely or partially to a depressor response. The pressor action of epinephrine is usually increased by the presence of ephedrine although in some cases it may be diminished (1). Lewis (2) has recently shown that several N-substituted hexylamines potentiate the pressor action of epinephrine, ephedrine, phenylethylamine, and cyclohexylethylmethylamine. A diminishing pressor response to repeated administrations (tachyphylaxis) occurs with a large number of sympathomimetic amines. Cross tachyphylaxis can also occur. Ephedrine has been shown to prevent the pressor action of some of the aliphatic amines thus allowing a depressor action to appear (3). Hambourger and Jamieson (4) showed that β-(*p*-ethylphenyl) ethylmethylamine reversed the pressor action of phenylethylamine and other similar compounds.

This report concerns another example of inversion of the pressor response; the pressor action of Vonedrine (β-phenyl-*n*-propylmethylamine) is converted to a depressor action by the presence of Paredrine (β-(*p*-hydroxyphenyl) isopropylmethylamine). Paredrine has an action similar in most respects to that of ephedrine (5, 6), lacking, however, the marked central stimulant action. Tachyphylaxis to the pressor response develops if the doses are rapidly re-

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† This study was supported in part by a grant from Eli Lilly and Co. The drugs used in this study were kindly supplied by the following: Wm. S. Merrell Co. (Vonedrine HCl), Smith, Kline and French (Paredrine HBr), Frederick Stearns and Co. (Sympatol HCl), Sharpe and Dohme (Propadrine HCl), Ciba Pharmaceutical Products, Inc. (Priscol HCl), and Lederle Laboratories (Heparin).

peated (7). Vonedrine, a volatile amine which has been proposed as a nasal vasoconstrictor, also has an action similar to ephedrine (8). Tachyphylaxis to the pressor action of this drug has not been described.

EXPERIMENTAL

Dogs lightly anesthetized with morphine and ether were used throughout. Blood pressure was recorded from the carotid or femoral artery by means of a mercury manometer or a Hamilton manometer (9). Blood flow was measured in the hind leg or head by inserting a Gregg-Shipley Rotameter (10) into the appropriate artery following the administration of heparin. The height of the float in the Rotameter was recorded on the kymograph by an electro-mechanical system. Atropine (0.5 mg. per Kg.) was administered to the animals to abolish the vagal reflex and other postganglionic cholinergic effects. The amines were used in the form of their water-soluble salts and all doses are given on a milligram per kilogram basis.

after Paredrine producing a marked fall in blood pressure associated with active peripheral vasodilation. If the dose of Paredrine was equal milligram for milligram with the dose of Vonedrine no pressor action of the Vonedrine was apparent. If, however, the dose of Paredrine was one-half to one-third that of the Vonedrine then Vonedrine produced a slight rise in pressure after the primary fall. With 1 mg. of Paredrine this reversal action persisted from one to two hours. Repeated doses of Vonedrine alone produced a diminishing pressor response (tachyphylaxis) but in no case did a depressor action become apparent.

At least two distinct mechanisms should be involved in this reversal of Vonedrine by Paredrine since the pressor action of Vonedrine is due both to cardiac stimulation and peripheral vasoconstriction. That Paredrine converts the vasoconstrictor action of Vonedrine to vasodilation is apparent in the blood flow studies. Vonedrine normally increased the heart rate in the atropinized dog during the pressor action but after Paredrine this increase in heart rate did not occur. This may be taken as evidence that the cardiac stimulant

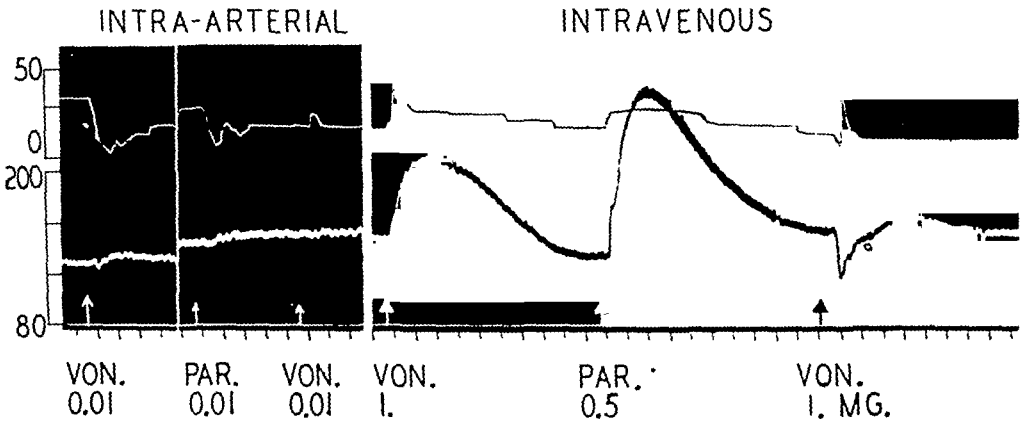

Fig. 1.—Carotid Blood Pressure and Femoral Blood Flow.

Dog, male, 13 Kg., Upper: Blood flow in cc. per minute. Center: Mean arterial blood pressure in mm. of Hg. Lower: Base line (at 80 mm. Hg) and time signals at one-minute intervals. At the first break in the record fifteen minutes and at the second break sixty minutes have been deleted. For the intra-arterial administrations the compounds were injected in a volume of 0.1 cc. into the peripheral side of the flowmeter. Intravenous administrations were made into the femoral vein. Note the active vasodilation produced by the Vonedrine after Paredrine. This dilation is shown by the increased blood flow without any change in arterial pressure after intra-arterial injection. On intravenous administration the dilation is more apparent as the flow increases during the marked fall in pressure. The increased blood flow which occurs with the first intravenous doses of Vonedrine and Paredrine is a passive increase due to the high arterial pressure.

RESULTS AND DISCUSSION

The observation that Paredrine reversed the pressor action of Vonedrine was first made during the course of another study on the sympathomimetic amines. It was observed that doses of 1 to 3 mg. of Vonedrine lowered the blood pressure in an animal which had received 1 mg. of Paredrine twenty minutes before. This observation was then confirmed in several other animals. Figure 1 shows the characteristics of this reversal effect, Vonedrine

action of Vonedrine is inhibited by the Paredrine. The effect of Vonedrine after Paredrine probably represents the algebraic sum of the diminished cardiac stimulation and the active peripheral vasodilation. When equal doses of the two compounds are used the cardiac stimulant action of the Paredrine is completely inhibited, therefore only a depressor response due to the vasodilation appears. When the dose of Paredrine is less than the dose of Vonedrine the depressor phase is followed by a slight pressor response due to only partial inhibition of the

Vonedrine cardiac stimulation by the Paredrine.

A number of other amines were tested as to their influence on Vonedrine. These included tyramine (β-(p-hydroxyphenyl) ethylamine), Sympatol (β-(p-hydroxyphenyl) ethanolmethylamine), Propadrine (β-phenyl isopropanolamine), ephedrine, and epinephrine. None of these compounds inhibited or reversed Vonedrine and none of them restored the Vonedrine pressor action after Paredrine. Paredrine had no reversing action on any of these compounds. The Paredrine pressor action was unchanged following Vonedrine.

An inversion of the pressor action of the sympathomimetic amines may occur if the pressor response is removed by an antipressor agent, and if a normally masked depressor effect is present. Epinephrine reversal by the adrenolytic agents is due to this type of action. Benzylimidazoline hydrochloride (Priscol-Ciba), a sympathomimetic antipressor agent, has been shown to selectively block the pressor action of the amines without influencing their depressor action (11). Priscol effectively blocks the pressor action of Vonedrine; however, it does not produce Vonedrine reversal (11). This would indicate that Vonedrine does not normally possess a depressor action.

Some of the aliphatic amines have a depressor action which becomes apparent only after their pressor action is diminished through repeated doses or through the previous administration of ephedrine (3). Priscol does not bring out this depressor action of the aliphatic amines, but if the depressor action is present Priscol does not influence it (11). After the depressor action of Vonedrine had been elicited by Paredrine, administration of Priscol appeared to prevent the fall in pressure produced by the Vonedrine. Since Priscol has been shown to have no blocking action on sympathomimetic vasodilation this might indicate that the vasodilation produced by Vonedrine is not of the usual type.

This interaction of Paredrine and Vonedrine illustrates one of the chief difficulties in comparative studies on the sympathomimetic amines. The fact that one amine may greatly modify the response of another, which has often been overlooked or ignored, has given rise to some of the very divergent results reported in the literature. In comparing the activities of the sympathomimetic amines probably not more than one dose of the long-acting drugs should be used in any one animal in any one day. Even some of the so-called short-acting amines have actions which persist much longer than is shown by measurements of the mean blood pressure. This has been demonstrated by Kleinberg, *et al.* (12), who showed that epinephrine increases the intramuscular pressure for periods exceeding two hours.

SUMMARY

1. Paredrine reverses the pressor action of Vonedrine, changing its vasoconstrictor action to a vasodilator effect.

2. Epinephrine, ephedrine, Propadrine, Sympatol, and tyramine do not reverse Vonedrine and neither are any of them reversed by Paradrine.

3. This inversion of the Vonedrine pressor action by Paredrine appears to be an actual reversal of action and not an unmasking of a normally present response.

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Book Reviews

Acetanilid—A Critical Bibliographic Review, by MARTIN GROSS. Hillhouse Press, New Haven, Conn., 1946. 155 pp. 15 x 23.5 cm. Price $3.00.

This critique of acetanilid is volume one of a proposed series of monographs to be published by the Institute for the Study of Analgesic and Sedative Drugs. The topics discussed in this monograph are the history, physico-chemical properties, metabolism, therapeutic uses, pharmacology, toxicology, tolerance, habituation and addiction, and other factors pertaining to acetanilid medication.

The author evidently made a quantitatively thorough literature search as evidenced by the 763 references cited. It is the "critical" feature of the bibliography with which many will take issue. Most of the discussion centers around the toxic and undesirable properties of acetanilid. It is distinctly noticeable that the author is highly critical of papers showing the drug to be toxic while papers reflecting the opposite viewpoint are glossed over. A good example of this is the author's reaction to a paper by Kebler (cf. pp. 58–60). Kebler sent a questionnaire to 925 physicians and the 400 replying indicated 17 deaths attributable to acetanilid. The author's own survey covering the period showed 18 deaths from acetanilid and yet the author used nearly 3 pages to try to explain away Kebler's findings. To the reviewer, if there were 17 deaths resulting from acetanilid during the period 1886 to 1907, acetanilid is not a completely safe drug, even if the statistical approach of Kebler were not entirely sound.

The reviewer takes strong issue with the statement on page 59: "Opinions on the safety of a drug may be based on animal experiments, on the frequency with which toxic symptoms are observed in man, or on the magnitude of the difference between the lethal and therapeutic doses; *but the most compelling evidence is the record of destruction of human life by the drug*" (italics the reviewer's). The additional statement by the author that "there is no assurance that all or any large proportion of fatalities are reported" is not only an understatement but makes the preceding statement even more amazing. Certainly with a drug of the character of acetanilid, it is the daily exposure that is the important element. The reviewer is of the opinion that there are many cases of chronic acetanilid "poisoning" in which the general health of the person is improved by the withdrawal of acetanilid and yet the cases are not recorded in the literature.

The completeness of the documentation and the evidence of needed careful studies by more modern methods make the book worth while if it is put to genuine scientific purposes and not used as an apology.—MELVIN W. GREEN.

Richter's Chemistry of the Carbon Compounds, Volume III. Edited by RICHARD ANSCHÜTZ and translated by A. J. MEE. Elsevier Publishing Company, New York, 1946. xviii + 794 pp. 13.5 x 21.5 cm. Price $15.00.

Volume III of *Richter*, which is concerned with the aromatic compounds, is so well known that it scarcely needs a review. Organic chemists are grateful to the publisher for making available this volume in spite of war conditions. Particularly helpful are the references to the original literature rather than *Chemisches Zentralblatt*.

Pharmacists will be disappointed, however, in the poverty of information on recent medicinal agents. Sulfanilamide is the only sulfonamide mentioned and it rates about one-third of a page. Nothing is said concerning the chemistry of the multiplicity of biochemical agents having a steroid nucleus. Procaine is barely mentioned and ephedrine is the only sympathomimetic drug considered.

In spite of pharmaceutical shortcomings, Richter will continue to fill a valuable place on the reference shelves of all interested in the many facets of organic chemistry—MELVIN W. GREEN.

Physical Methods of Organic Chemistry. Volume II. Edited by ARNOLD WEISSBERGER. Interscience Publishers, Inc., New York, 1946. vii + 629 pp. 23 x 14.5 cm. Price $8.50.

Volume II of this reference book contains chapters on: Spectroscopy and spectrophotometry; colorimetry, photometric analysis, and fluorometry; polarimetry; determination of dipole moments; conductometry; potentiometry; polarography; determination of magnetic susceptibility; determination of radioactivity; and mass spectrometry.

The subjects covered by this volume are so diversified that it is difficult to evaluate the book as a whole. One of the basic impressions that the reader obtains is that there is too much emphasis on instrumentation and not enough application to organic chemistry.

The sections on spectroscopy and spectrophotometry are very helpful. One questions the value of a separate chapter on photometry since there is considerable duplication with the spectroscopy chapter. The portions dealing with infrared spectroscopy and the Raman effect are much too short.

Most of the description of optical devices for polarimetric measurements are generally available. The section on nonvisual and spectropolarimetry are unusually good, however.

The chapter on conductometric titrations includes both a section on conductometric titrations and a

section on electrophoresis. In Dr. Michaelis' chapter on potentiometry, potentiometric titrations, pH, and oxidation-reduction potentials are described.

Dr. Müller's chapter on polarography is replete with examples of application to organic chemistry. Not only the qualitative and quantitative aspects are described, but the author gives considerable attention to the study of tautomerism, polymerization, and related phenomena.

The section on radioactivity contains no application, but is limited to methodology. The section on mass spectroscopy gives considerable valuable information to the organic chemist. The techniques are too cumbersome and costly for the average laboratory, however.

The two volumes of this excellent reference book should open new vistas to the organic chemist. The physical-chemical approach to organic chemistry is throwing new light on many old and new organic problems and *Physical Methods of Organic Chemistry* will prove to be a helpful guide to this rapidly expanding field.

For a review of Volume I of this book the reader is referred to THIS JOURNAL, 35, 32(1946).—MELVIN W. GREEN.

Introduction to Emulsions, by GEORGE M. SUTHEIM. Chemical Publishing Company, Brooklyn, 1946. vii + 260 pp. 10.5 x 17 cm. 22 illus. Price $4.75.

The book is divided into six chapters dealing with the theoretical foundation, physical chemistry and chemistry of emulsifying agents, and formation, properties, and applications of emulsions. The basic considerations, underlying principles, and theories are well presented, and offer a good foundation for one who is interested in the fundamentals of emulsification.

The background is less pharmaceutical than commercial or technical, little actual consideration being given to the operation of preparing extemporaneous emulsions as practiced by the pharmacist of today. No mention is made of the use of Pharmagel (Gelatin) as developed by Tice, nor is there any reference or illustration to the very popular and efficient hand homogenizer.

The book contains an extensive table of emulsifying agents and pertinent facts relating to them. These agents are primarily of the newer type commonly referred to as wetting agents, mostly of commercial and industrial interest. A comprehensive and valuable bibliography is also included.

The book is a worthy addition to the library of anyone interested in the principles of emulsification. —ADLEY B. NICHOLS.

Organic Qualitative Microanalysis, by FRANK SCHNEIDER. John Wiley and Sons, New York, 1946. iv + 218 pp. 136 figs. 13.5 x 21.5 cm. Price $3.50.

Microchemistry, now a recognized specialty in the chemical field, has progressed very rapidly during the past decade. Qualitative organic microchemistry has been the most backward in the micro field, while quantitative organic microchemistry has probably progressed the furthest.

Professor Schneider has written this book to be used as a textbook and laboratory manual by organic chemistry students interested in the systematic identification of organic compounds. The system follows very closely the Mulliken–Huntress classification, but aims to use a sample about 1/100 the normal size.

Methods of purifying samples containing mixtures, determining physical constants of pure organic compounds, and preparing characteristic identifying derivatives are given. Most tests and techniques are described in considerable detail and with adequate diagrams to facilitate the work.

In the hands of an instructor familiar with microprocedure, this book no doubt will serve as an excellent guide to a fascinating field. The novice and research worker, using such testing only occasionally, will probably find the fact that such careful attention must be given to details of the technique a source of discouragement.—MELVIN W. GREEN.

Colloids—Their Properties and Applications, by A. G. WARD. Interscience Publishers, Incorporated, New York, 1946. 133 pp. 11 x 18 cm. Price $1.75.

This little volume is not intended to be a complete treatise on the colloidal state, but rather to review briefly colloidal chemistry and its modern application in simple, quasi-technical language.

The book is divided into three parts with a total of 17 chapters. Part I deals with the nature of the colloidal state and discusses atomic structure, the physics of surfaces and the physical chemistry of the colloidal state.

Part II deals with colloidal systems. Here are discussed very succinctly the lyophobic and lyophilic systems as well as gels, pastes, emulsions, foams, dusts, smokes, and fogs.

The third part is concerned with the application of colloidal theory to rubber, cellulose technology, proteins, clay, paints and varnishes, detergency, and to life processes.

While not adequate as a textbook in a course in colloidal chemistry at the university level, this little book is fascinating to read and well worth while as a quick source of review.—MELVIN W. GREEN.

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The Colorimetric Assay of Digitoxin by the Modified Raymond Method*

By ROBERT C. ANDERSON and K. K. CHEN†

Digitoxin, when treated in dilute solution with *m*-dinitrobenzene and sodium hydroxide, produces a blue color which can be estimated accurately in a photoelectric colorimeter. The method is particularly useful in the assay of tablets and ampuls of digitoxin. Necessary equipment employed in the test is described. A comparison of the results by this method and those by the U. S. P. cat method is made. The color developed is not specific, but it serves as a quantitative measure if admixture with other cardiac glycosides is not anticipated.

NO SOONER did Gold (1) demonstrate the complete absorption of digitoxin following oral administration in human beings than it became widely used in the treatment of cardiac failures. Additional advantages of digitoxin over galenical preparations are constancy of action because it is a single chemical entity, and absence of constituents irritating to the stomach, so that nausea and vomiting are less apt to occur. Besides, this development represents a phase of medical progress, since more and more active principles have replaced the extractives of plants or animal organs from which the former are isolated.

The assay of digitoxin becomes a new problem. Obviously, the determination of physical constants, such as the melting point and optical rotation, is not sufficient to establish its purity and potency. To insure uniformity of the product, the U. S. P. biological assay by the cat method (2) is undoubtedly the most reliable. Unfortunately, this method is not only costly and time-consuming, but it is often handicapped by a shortage of cats. A chemical or colorimetric method is highly desired, particularly for checking the intermediate steps of manufacture, such as need of recrystallization and assay of the tablet granulations. Bell and Krantz (3) have already elaborated a colorimetric method based on the Baljet reaction (4), and have advocated its use in assaying digitoxin.

During the last few years, we have been

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‡ Presented to the Scientific Section, A. PH. A., Pittsburgh meeting, August, 1946.
† We are indebted to Messrs. Chester C. Hargreaves and William T. Winchester for their work on the cat assays; also to Mr. Fred A. Rice, Jr., for his assistance, and to Mr. E. Brown Robbins for his statistical help and advice.

investigating the Raymond reaction of *m*-dinitrobenzene (5, 6) with digitoxin. The ultimate color is blue and is easy to measure and compare with that of the standard, although it disappears quickly. The color reaction is believed to be caused by the lactone ring of the glycoside. It has advantages and disadvantages similar to those of the Bell-Krantz method. Numerous comparisons were made with the Bell-Krantz, U. S. P. biological, and the Raymond methods. The majority of the lots of digitoxin were manufactured in our own plant. One specimen was generously supplied to us by Dr. Walter A. Jacobs, Rockefeller Institute for Medical Research, New York City; and another by Professor R. P. Linstead, now Director of the Chemical Research Laboratory, Teddington, England, through the courtesy of Dr. Otto Krayer, Harvard Medical School, Boston.

EXPERIMENTAL

Procedure.—The reagents needed are a 1% solution of *m*-dinitrobenzene in absolute ethanol and a 20% solution of sodium hydroxide. For the test, 1 cc. of *m*-dinitrobenzene solution is added to 10 cc. of dilute digitoxin solution in approximately 47.5% ethanol and placed in an ice bath. After five to ten minutes, 2 cc. of sodium hydroxide solution is added and the mixture returned to the ice bath after thorough mixing. The indigo-blue color produced is read exactly five minutes after the addition of sodium hydroxide. For this test, a Cenco-Sheard-Sanford "Photelometer," a water blank, and No. C orange filter were employed. A portable cooling unit, thermostatically controlled, provided a bath maintained at 0° C. The maximal color develops in five minutes and fades fairly rapidly.

To construct a standard curve, the following dilutions are recommended. Volumes of 0.75 cc., 1.0 cc., and 1.25 cc. of 1:1000 digitoxin solution are diluted to 50 cc. with 47.5% ethanol, so that concentrations of 0.015, 0.020, and 0.025 mg. per cc. are obtained. The blue color in each dilution is produced as described above. The known amounts of digitoxin in the solutions are plotted as ordinates on the equal-division axis of semilogarithmic paper against the "Photelometer" readings as abscissas on the logarithmic scale. Where micro-cells are available, the quantities may be reduced, so that 2 cc. of dilute digitoxin solution, 0.2 cc. of *m*-dinitrobenzene reagent, and 0.4 cc. of sodium hydroxide are used.

To perform the Bell-Krantz test, a Beckman Quartz Spectrophotometer was used in place of the Electrophotometer of the Fisher Scientific Company. The transmissions were compared at 525 mμ. in a 1-cm. cell. The rest of the technique was the same as published.

Results with Unknown Solutions of Digitoxin.—To check the validity of each method, unknown dilutions of a standard lot of digitoxin were prepared and assayed. The results are shown in Table I.

TABLE I.—RESULTS WITH UNKNOWN SOLUTIONS OF DIGITOXIN

Theoretic Concentration %	Observed Concentration Raymond Method, %	Observed Concentration Bell-Krantz Method, %
0.100	0.103	0.105
0.075	0.073	0.078
0.090	0.089	0.092

There is indeed a close agreement between the results of the two methods. The differences between theoretical and observed concentrations are very small. These data clearly indicate that the intensity of color in each method is directly proportional to the concentration of the solution. They attest the accuracy of the curves of known concentrations.

Repeated Tests on the Same Lot of Digitoxin.—For any potent product like digitoxin, it is our practice to set aside a lot to serve as a "House Reference Standard." Such a standard would be assigned a potency of 100%. The potencies of all other lots are based on this figure. Our lot No. 1 was our reference standard. It met all the physical specifications, and by the U. S. P. cat method assayed 2.14 units per mg. The sample has been repeatedly tested colorimetrically by both the Raymond and Bell-Krantz methods, and the results, listed in Table II, have been computed from the predetermined curves. The greatest experimental errors were −3.2% and +2.6%, with the Raymond method; and −4.5% and +4.2%, with the Bell-Krantz method. It is perhaps fair to say that the limits of error by each method is within ±5%.

TABLE II.—RESULTS ON LOT NO. 1[a]

Raymond Method, %	Bell-Krantz Method, %
99.2	99.4
98.7	101.8
102.6	103.9
96.8	95.5
100.7	104.2

[a] "House Reference Standard."

Assay of Experimental and Manufactured Lots.—The relative potencies of 21 lots of digitoxin as determined colorimetrically are compared in Table III. The results by the U. S. P. cat method are also included. Lot No. 1 was the standard for all other lots. It should be noted that the results by the biological method on the one hand and by the two colorimetric methods on the other agree satisfactorily; that is, they are all within the customary

TABLE III.—ASSAY OF VARIOUS LOTS OF DIGITOXIN BY THREE METHODS

Lot No.	Potency U. S. P. Cat Assay, %	Raymond Method, %	Bell–Krantz Method, %
1[a]	100	100	100
2	92	98	96
3	96	94	91
4	89	99	94
5	101	89	98
6	89	91	99
7	106	97	106
8	103	96	104
9	89	84	101
10	96	103	104
11	99	98	102
12	111	103	100
13	106	92	98
14	102	99	102
15	101	97	106
16	94	96	104
17	110	103	105
18	87	99	101
19	96	101	104
20	92	80	98
21	90	82	92

[a] "House Reference Standard."

U. S. P. limits of tolerance of ±20%. Out of 21 lots, the maximal variations by the Raymond method from the biological method are +12% and −14%; and those by the Bell–Krantz method from the biological method are +14% and −11%. The extreme differences between the readings by the two colorimetric methods do not exceed 18% (lot No. 20).

Assay of Tablets.—Thirty tablets, 0.2 mg. digitoxin per tablet, are triturated and transferred to a 60-cc. centrifuge tube. Sixty cc. of 80% ethanol are added and the tube is securely stoppered. It is then placed on an extractor built in our own laboratory, which rotates approximately 20 revolutions per minute. The mixture is allowed to extract overnight and then centrifuged. A volume of 5 cc. of the supernatant liquid is pipetted to a 25-cc volumetric flask and diluted to the mark with 47.5% ethanol. The color is developed as outlined above and theoretically should contain 0.02 mg. per cc. For 0.1-mg. tablets, a volume of 10 cc. of supernatant liquid is diluted to 25 cc., the theoretical quantity of the final dilution remaining 0.02 mg. per cc. Tablet granulations, namely, the powder to be made into tablets, are assayed in a quantity equivalent to the weight of 30 tablets.

Eleven lots of digitoxin tablets of our own make were assayed by the Raymond method. The results as enumerated in Table IV are unquestionably acceptable—without exception.

For comparative purposes, the same lots of tablets were assayed by the Bell–Krantz method. It was soon observed that if absolute methanol was used for extraction the readings would be 15% to 32% too low. Addition of 1 part water to 3 parts methanol, resulting in 75% methanol, readily affects complete recovery of digitoxin. The use of 75% methanol may eliminate any subsequent turbidity as experienced by Bell and Krantz (3), because this dilution will not extract an appreciable amount of the lubricant, such as magnesium stearate. It is also important that extraction should not exceed twenty to thirty minutes, as advised by the authors. Apparently, prolonged extraction, say overnight, would include a sufficient amount of lactose present in the tablets, which will react with picric acid and give rise to a falsely high content of digitoxin. After these precautions are taken, concordant results may be obtained as illustrated in Table IV. In contrast with the Bell–Krantz method, the Raymond method encounters no interference with lactose in spite of prolonged extraction.

It is conceded that neither the Raymond nor the Bell–Krantz method is specific for digitoxin. Similar color reactions take place when other cardiac glycosides are substituted. However, both methods can facilitate the manufacture of digitoxin tablets, provided no interfering ingredient is added. Obviously, the methods are also applicable to ampuls, as we have already tested them several times and found them satisfactory.

TABLE IV.—ASSAY OF DIGITOXIN TABLETS

Tablet Lot No.	Digitoxin Found Raymond Method, %	Bell–Krantz Method, %
1	96	101
2	98	104
3	103	98
4	95	100
5	93	96
6	100	96
7	95	96
8	100	96
9	106	92
10	101	103
11	103	94

CONCLUSIONS

1. Two colorimetric methods for the assay of digitoxin have been compared. The results on 21 lots of crystalline digitoxin agree in general with those by the U. S. P. cat assay.

2. Eleven lots of digitoxin tablets have been assayed by the same procedures with good agreement.

3. Both methods are accurate, inexpensive, and easily handled, provided certain precautions are observed.

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Studies on Pyrogens. II. Some Factors Influencing the Evaluation of Pyrogen Tests*

By HANS MOLITOR, MARY E. GUNDEL, SAMUEL KUNA, and WALTHER H. OTT†

By application of a technique making use of thermocouples for recording body temperatures, more than 6000 pyrogen tests involving over 19,000 rabbits have been conducted. Biometrical analysis of data obtained from these tests and progress toward the development of a quantitative method for the determination of pyrogens using a reference standard and standardized test animals are described.

SINCE the demonstration by Seibert (1, 2) that the occasional elevation of body temperature following intravenous injection of aqueous solutions of medicinal agents, as well as distilled water, is due to pyrogenic substances of bacterial origin rather than to a specific action of the drug itself, increasing attention has been paid to the control and elimination of these contaminants. The combined efforts of numerous investigators in government and industrial laboratories (3, 4) and in hospitals (5), coordinated by the Sub-Committee on Bioassays of the Revision Committee of the U. S. Pharmacopœia, have greatly advanced our knowledge in this field and have resulted in the institution of an official pyrogen assay in the U. S. Pharmacopœia. This test will detect the presence of pyrogens, but it does not permit their quantitative determination. Such information, although unnecessary for the safe clinical use of the material, is highly desirable for research purposes. The chemist working on the purification and structural identification of pyrogens, the pharmacist trying to develop processes for their removal, the pharmacologist investigating their mode and 'e of action, and the clinician studying 'r toxicological significance—all require rmation as to the actual amounts pres- Such a quantitative test will for a time be based on a bioassay procedure. le chemical test methods have been suggested, such as the permanganate titration for organic matter, they are as yet of no practical value because of their lack of sensitivity and specificity; only after the chemical nature of pyrogens is better known may we expect the development of adequate and specific chemical assays. Even then, however, there will be need for a biological test since already there is ample evidence that different types of microorganisms produce pyrogenic substances which differ widely in their chemical composition (6, 7). There is also some indication that pyrogens of different origin possess different pharmacological and toxicological properties, some of them being capable of producing fever immediately, others only after a certain lag (8), while still others are likely to reduce the body temperature before causing a rise (9).

In a previous communication from this laboratory (10), a technique has been described which makes use of thermocouples for the recording of body temperatures instead of the customary rectal mercury thermometer. This results not only in a considerable saving of time but also in more physiological test conditions since it dispenses with the repeated handling of the test animals. During the last two years more than 6000 pyrogen tests involving over 19,000 rabbits have been conducted in our laboratory with this technique with highly satisfactory results. Biometrical analysis of these data as well as continued research on a quantitative assay procedure has suggested possible improvements in the customary assay which will be presented in the following pages.

It is well known that the accuracy of a biological assay primarily depends upon the following factors: (*a*) a constant and dependable reference standard as a basis for the comparison of the unknown substances; (*b*) a standardized test animal, whose deviations from the norm are small and known;

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The valuable technical assistance of Messrs. Charles F. and Gordon B. Thomas is gratefully acknowledged.

(*c*) a valid biometrical procedure for the evaluation of results.

In spite of the large amount of research which led to the establishment of the presently used official pyrogen assay (11) much remains to be done in order to transform this excellent qualitative method into a quantitative one; indeed, it is doubtful whether it will ever be possible to devise a pyrogen test which in accuracy can favorably compare with such standard procedures as the U. S. P. digitalis, ergot, or insulin tests. However, it should be emphasized again that the shortcomings of the present official assay become manifest only if the test is used for strictly quantitative determinations, a purpose for which it was originally not intended.

One of the greatest obstacles in making this test more quantitative is the difficulty in standardizing the test animal. The rabbit, which at present is universally used for this purpose, possesses a notoriously labile temperature regulation. The wide variations in its body temperature reported by different investigators, ranging from an extreme low of 36.6° (12) to an extreme high of 41° (13) are ample proof of the difficulties involved, even though the reported ranges for the *average* high and low temperatures are considerably narrower, e.g., 38.8–39.7° (14); 39.07–39.85° (13); 39.4–39.9° (15). In addition to the wide differences between individual animals there are also considerable daily and hourly temperature fluctuations in the same animal, largely under the influence of nervous factors.

That the temperature-regulating mechanism should be sensitive to nervous stimuli, such as excitement due to handling, is not surprising, since it is well known that in this species other centrally regulated functions, e.g., water metabolism and vascular reflexes, react similarly to stimuli of this nature (16, 17).

MATERIALS AND METHODS

Male albino rabbits obtained from various sources and weighing 1 to 3 Kg. were used. In general the method of conducting the pyrogen test was similar to that described in the U. S. Pharmacopœia XII. However, in the majority of experiments the animals were kept for the entire duration of the test in specially constructed boxes, and stationary thermocouples were used for recording the rectal temperature, following the technique recently described by one of us (10).

The pyrogen concentrate used as a standard in these tests was prepared from *Pseudomonas aeruginosa* (*bacillus pyocyaneus*) according to the technique of Welch, *et al.* (3). A minimum pyrogenic dose was defined as that amount of the standard which was necessary to cause an average temperature rise of 0.5° in rabbits, when injected intravenously. The amount of our concentrate required to produce this effect was usually 1 cc. per Kg. of a dilution of 1:5000.

Experimental

Previously (10) it has been shown that the body temperature of rabbits which are kept quietly in boxes is generally lower than that of animals disturbed by repeated removal from their cages. However, these experiments were not designed to determine whether or not the consistently lower temperature of undisturbed animals was specifically due to the absence of handling or excitement. It was therefore decided to study the influence of these factors by keeping continuous records of a rabbit's body temperature while exposing the animal to a variety of nervous stimuli.

The iron-constantan thermocouples used were of the flexible, rubber-covered type previously described (10). The recording device used in these tests was designed and constructed by Messrs. Michael Kniazuk and Omer S. Lucier of our laboratory and consisted of a conventional system of a reference thermocouple with a second thermocouple and a reflection type galvanometer as an indicator. In order to obtain a continuous record of the temperature, the galvanometer spot was directed to a photoelectric cell, which was so arranged that it automatically followed the galvanometer deflection and traced its position on a moving-paper chart. A deflection of 2 cm./degree C. was found adequate. The response time of the system depended primarily on the heating time of the thermocouple and movement of the galvanometer. Both were found to be practically negligible for this work.

A tracing typical of the effect of handling and of inserting a rectal thermometer is reproduced in Fig. 1. Insertion of the thermometer usually is followed within sixty to ninety seconds by a temperature rise up to 0.7°. The temperature remains elevated for about ten to thirty minutes after which time it slowly returns to normal.

Figure 2 shows the average temperature curve of a group of rabbits taken in the manner described in our previous paper (10) in animals placed in boxes and, after insertion of rectal thermocouples, left undisturbed for several hours. It can be seen that under these conditions the temperature declined during the thirty to forty-five minutes following insertion of the thermocouple; when the lowest point had been reached a slight increase followed, after which the temperature remained relatively constant

This "basal" temperature was reached about sixty to ninety minutes after the start and was always considerably below the starting temperature.

These results are in agreement with those obtained with continuous recording of the body temperature. They indicate that temperature readings, taken one to two minutes after the insertion of a thermometer (or thermocouple), are on an average 0.2 to 0.3° higher than the "basal" temperature and that it takes about ten to thirty minutes after each handling before the body temperature has returned to the lower level. Obviously, there are differences among rabbits in the degree to which they react to handling; furthermore, the same rabbit is likely to show a progressive decline in reaction as he becomes used to the procedure.

Since the average basal temperature of undisturbed rabbits is well below that of rabbits tested in the usual manner (10), it was of interest to examine the influence of different starting temperatures on the response to a pyrogenic agent. Just as elevated temperature levels are best suited for the demonstration of an effect of temperature lowering agents, it would be expected that a low starting temperature would favor a temperature increase. An examination of Fig. 3 will show that these expectations materialized. The maximum rise following injection of a pyrogenic material was

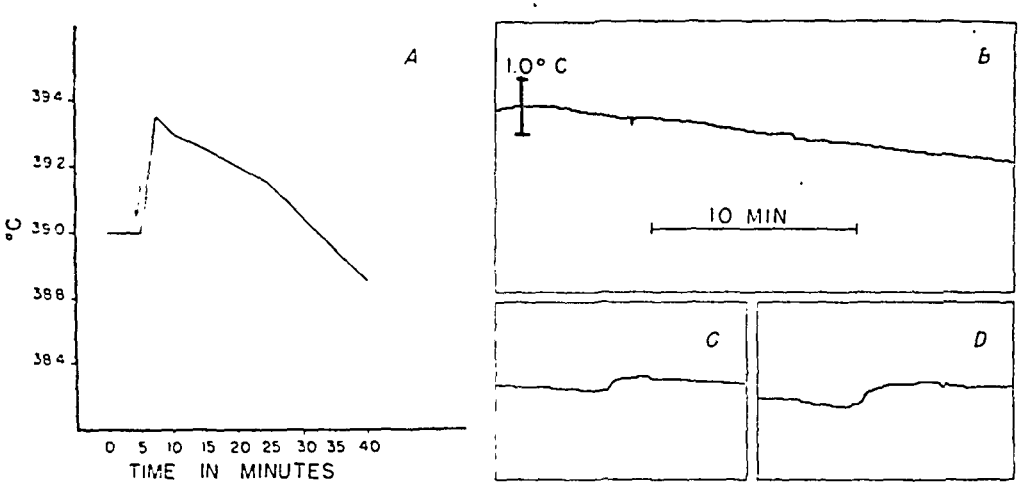

Fig 1 —Influence of handling on temperature of rabbits. Continuous temperature recordings taken from rectally inserted thermocouples.

A—Temperature rise following mock insertion of a rectal thermometer (with rectal thermocouple remaining in place).

B—Temperature decline resulting from freedom of disturbances. (Rabbit kept in box.)

C and *D*—Effect of slight movements of the rectal thermocouple, simulating insertion of a thermometer

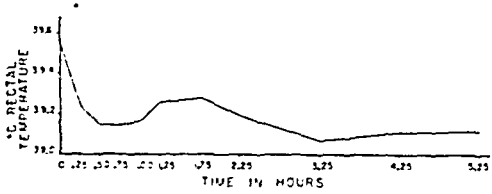

Fig. 2.—Curve showing the change in rectal temperature in a group of 34 rabbits during five hours from the time they were placed in boxes. The average rectal temperature of these rabbits taken with clinical thermometers approximately forty minutes prior to being placed in the boxes was 39.53°.

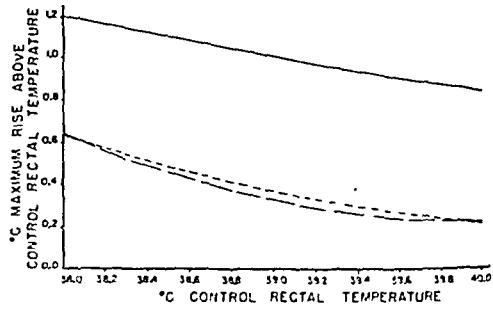

Fig. 3.—Curves showing the relation between control rectal temperature and maximum rise in rectal temperature in noninjected rabbits (dotted line) following intravenous injection of pyrogen-free saline (broken line) or of a standard dosage of pyrogen (solid line). The curve for the pyrogen standard ($Y = 23.69 - 0.9857X + 0.01036X^2$, where Y is maximum rise in ° C. and X is control temperature in ° C.) was constructed from the data of 624 individual tests and represents the average maximum rise observed in rabbits having the corresponding control temperatures. The curve for the pyrogen-free water, $Y = 175.65 - 8.782X + 0.1099X^2$, was calculated from the data of 486 individual tests. The curve for the noninjected rabbits, $Y = 95.91 - 4.695X + 0.05758X^2$, was calculated from data of 731 individual tests. The control temperatures in these 1841 individual tests ranged from 37.1° to 41.6° in a normal frequency distribution with mean ±S. E. of 39.03 ± 0.014°.

inversely proportional to the starting temperature. Thus, the average maximum rise following 4 minimum pyrogenic doses increased from 0.9° at 39.6° to 1.1° at 38.4°. The establishment of an arbitrary limit of temperature variation, beyond which a temperature increase would be regarded as critical, is therefore justified only within a relatively narrow temperature range.

Since animals with a lower starting temperature show a more pronounced reaction to pyrogenic contaminants than those with higher starting temperatures, their use would appear to be preferable. In this case, however, the fixed maximum permissible rise of 0.6°, presently used in the U. S. P. assay, would have to be replaced with a variable limit, in order to obtain physiological equivalent conditions at both high and low initial temperatures. Thus, for example, a temperature rise of 0.8° in the zone of 38.0–38.3° might be approximately equivalent to one of 0.6° at 38.7–39.4° and one of 0.5° at 39.5–40.0°.

Another question is whether the normal fluctuations in body temperature are the same for various initial temperature levels or whether the apparently greater sensitivity of the temperature-regulating mechanism at lower levels is likely to cause wider spontaneous fluctuations at that level. An examination of our data (Fig. 3) indicates that the greater response to pyrogenic stimuli at the lower temperatures is not offset by the greater tendency to "spontaneous" fluctuation. It would therefore seem advantageous to select rabbits with low starting temperatures and to operate under experimental conditions which are likely to produce such a range.

The wide temperature variations reported by previous investigators were obtained under experimental conditions which did not exclude excitement due to handling. It was therefore of interest to study the basal temperature curves of completely undisturbed rabbits, since the elimination of excitement might result in temperatures sufficiently constant to permit quantitative comparisons on the basis of the normal temperature of each individual rabbit. It was found, however, that the daily temperature fluctuations of undisturbed animals, even though much smaller than those obtained with the usual methods of temperature measuring, were nevertheless still too wide to permit an increased accuracy through the standardization of individual animals. Consequently, it seems preferable to base all evaluations on an average group response and to compare this with the response of a group to a pyrogren standard of known potency. The criterion of an average group response is accepted biometrical practice, particularly when it is difficult to standardize individual animals.

While in a more or less qualitative assay designed primarily as a safety control the unfavorable reaction of even a single animal may be sufficient cause for rejection or repetition of the assay, it certainly is inadequate in a quantitative test. We have investigated whether the introduction of the principle of group response would improve the accuracy and convenience of the pyrogen test. The results of this analysis are summarized in Fig. 4 which shows the effect of increased numbers of test animals per group on the accuracy of the test. In this group of

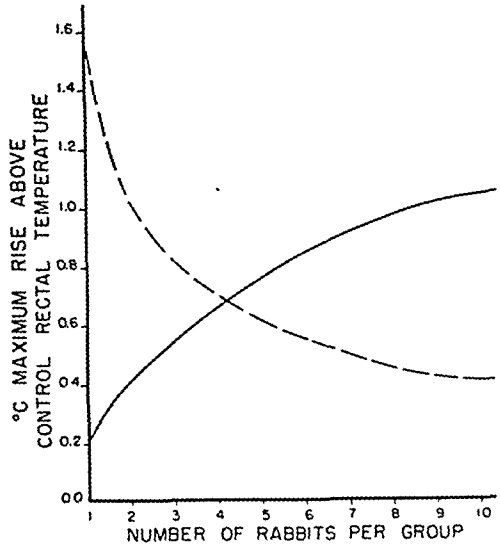

Fig. 4.—Curves showing the tendency for group averages progressively to differ less from the general average maximum rise in rectal temperature as the number of rabbits is increased per group. The average maximum rise in 105 tests following injection of 4 minimum pyrogenic doses per kilogram was 1.20°. The average maximum rise in 83 noninjected rabbits was 0.30°. The individual rises were successively regrouped into groups of different sizes as indicated and the average rises calculated for these groups. The solid curve was drawn through the lowest of the averages thus obtained with the data for the pyrogen-injected animals, and the broken line was drawn through the highest averages found with each of the different sized groups of untreated rabbits.

83 rabbits, the lowest temperature rise following injection of a standardized amount of pyrogen was 0.2° while the greatest rise in temperature of an untreated rabbit when compared with his starting temperature was 1.6°. When the evaluation was based on the average of a group of 3 rabbits, the tendency for the minimal average response to the pyrogen standard to be less than the maximum average fluctuation in a control group of similar size was greatly decreased. In groups of 5 animals or more the lowest average response to the pyrogen standard invariably exceeded the highest average fluctuation in temperature of any control group of similar size. It would therefore appear advantageous to use as a basis for quantitative evaluation the average response of a group of at least 3 to 5 animals and not give undue significance to a high reading occurring in only 1 or 2 animals.

It has been reported by previous authors (18) that there appeared to be little or no quantitative relationship between the dose of the injected pyrogen and the fever response in an individual rabbit.

In our experiments we have found that the individual responses vary so widely that only by consideration of group averages could a direct relationship between the dose of pyrogen and the temperature response be detected. This relation appeared to exist provided the pyrogen dose did not exceed reasonable limits, say, 50 minimal pyrogenic doses. If, however, higher doses were injected, the effect was one of prolongation of the fever rather than of a higher peak reading (Fig. 5).

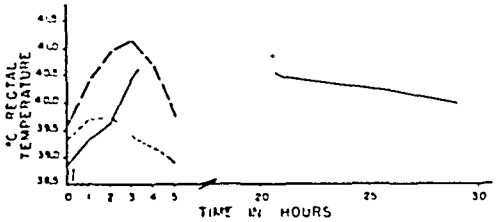

Fig. 5.—Curves showing the change in rectal temperature of rabbits following intravenous injection of standardized amounts of pyrogen solution. The injection was made immediately following the temperature reading at zero time. The lines represent the average temperatures of groups of rabbits as follows:

Solid line: 3 rabbits given 5000 minimum pyrogenic doses. Broken line: 5 rabbits given 80 minimum pyrogenic doses. Dotted line: 5 rabbits given 1 minimum pyrogenic dose.

From a study of the dosage response curve with graded levels of a pyrogen solution it was apparent that the average responses for groups of five rabbits were not uniform enough for satisfactory quantitative use (Experiments 1 to 3, Table I). In one experiment (Experiment 4, Table I), groups of 10 rabbits each were used, and the responses approached linearity when the temperature response was plotted against the logarithm of the dose. These tests were conducted before the influence of starting temperature had been elucidated. However, correction of the individual temperature rises on the basis of the corresponding starting temperature, using the sliding scale mentioned above, did not appreciably affect these results. Later experiments, described below, have indicated that the dose levels were too close together for satisfactory discrimination in these tests.

The first experiment conducted with a group of animals distributed equally on the basis of starting temperature among the different dosages showed a remarkable degree of linearity in the average responses, even though only three rabbits were used in each group (Experiment 5, Table I). These data indicate that a direct relationship might be expected over a wide range of starting temperatures, and that the response to pyrogen is greatest for the low starting temperatures as stated previously (see Fig. 3). Although allocation of the doses to groups equally balanced in advance on the basis of starting temperature is a time-consuming proposition, such a procedure is to be preferred to correction for such differences after the assay has been completed.

TABLE I.—AVERAGE MAXIMUM RISE IN RECTAL TEMPERATURE ABOVE STARTING TEMPERATURE OF RABBITS INJECTED INTRAVENOUSLY WITH GRADED LEVELS OF A STANDARD PYROGENIC SOLUTION

Experiment	Minimum Pyrogenic Doses, No./Kg.	No. Rabbits	Average Starting Temperature, °C.	Average Maximum Rise, °C.
1	1	5	39.32	0.52
	20	5	39.22	1.02
	30	5	39.32	1.48
	40	5	39.60	1.34
	60	5	39.64	1.44
	80	5	39.58	1.64
2	4	5	39.04	1.06
	6	5	39.30	0.96
	8	5	38.90	1.22
3	1	5	39.10	0.26
	2	5	39.10	0.82
	4	5	39.10	0.32
	10	5	39.02	0.72
4	1	10	39.04	0.43
	2	10	39.27	0.64
	4	10	39.19	0.56
	8	10	39.23	0.99
	10	10	39.13	1.10
5a	1.25	3	38.1	1.13
	2.50	3	38.3	1.27
	5.00	3	38.4	1.50
5b	1.25	3	38.7	0.63
	2.50	3	38.8	0.77
	5.00	3	38.8	0.80
5c	1.25	3	39.2	0.60
	2.50	3	39.2	0.77
	5.00	3	39.3	0.93

TABLE II.—AVERAGE TEMPERATURE RESPONSES FOLLOWING INJECTION OF GRADED LEVELS OF A DRY PYROGEN PREPARATION

Experiment	Dose of Pyrogen Preparation, Mg./Kg.	No. Rabbits	Average Starting Temperature, °C.	Average Maximum Rise, °C.
6	0.05	3	39.20	0.57
	0.1	3	38.87	0.87
	0.2	3	39.20	1.20
	0.5	3	39.17	1.60
	1.0	3	38.90	1.97
	2.0	3	39.00	2.13
7	1	3	39.33	1.07
	2	3	39.30	1.23
	8	3	39.40	1.20
8	1	3	39.23	0.83
	2	3	39.10	1.20
	8	3	39.00	1.53
9	0.01	3	38.93	0.23
	0.1	3	39.50	0.47
	1.0	3	39.00	1.07
10	0.05	3	39.03	0.33
	0.5	3	39.03	1.20
	5.0	3	39.03	2.23

Results of preliminary attempts to use a dry preparation of pyrogenic material as a standard are given in Table II. The absolute quantities listed have little significance, since they were largely influenced by the sodium chloride which was used as the dry diluent. It was estimated that 0.05 to 0.1 mg. of this material contained one minimum pyro-

genic dose of pyrogen. The object at this time was to investigate further the nature of the dosage-response curve.

The results (Table II) showed a reasonably satisfactory degree of linearity over a 500-fold change in dosage (0.01 mg. to 5 mg.). In fact it appears that for satisfactory discrimination between successive dosages a dose interval of 5- to 10-fold would be advisable. The average response varied from 0.5° to 1.0° maximum rise per 10-fold increase in dosage. Reference to the data in Table I shows that similar slopes were observed there, but that the dosages were generally too close together for practical purposes.

Robinson and Flusser (7) cited individual responses following injection of graded levels of different pyrogens. Calculated from their data the average responses varied from 0.6° to 0.9° maximum rise per 10-fold increase in dosage, which fall within the range of responses observed above. With this information it appears that application of any of the current biometrical assay methods, such as those described by Knudsen and Randall (18) might be successfully used in a quantitative assay of the pyrogen contents of biological materials.

DISCUSSION

Our experiments demonstrate the great influence of nervous stimuli on the body temperature of rabbits. Animals which are kept undisturbed and from which temperature readings are taken by means of a stationary rectal thermocouple show temperatures on an average 0.2° to 0.3° lower than those observed with the use of repeatedly inserted rectal mercury thermometers. Furthermore, the temperature of animals immediately after removal from their cages is on an average 0.5° higher than sixty to ninety minutes after undisturbed confinement to boxes or stalls. Even after the temperature of an animal has reached its basal level, it will again rise an average of 0.2° to 0.3° under the influence of each handling or insertion of a thermometer. This rise reaches its peak after sixty to ninety seconds and returns to normal within ten to thirty minutes.

An experiment in which the rectal temperature of a group of new, untreated rabbits was measured by means of stationary thermocouples during a period of five weeks showed that the average fluctuation between the temperature at the beginning of each test and that at the end of a three-hour observation period declined from a maximum of approximately 0.4° on the fourth day of the test to less than 0.05° after the twenty-fifth day (Fig. 6). This decline of 0.4° in the maximum fluctuation, due to handling, roughly corresponded to that of another group of new rabbits on which actual pyrogen tests were performed at two- to three-day intervals for a two-week period. In the latter group the maximum response to a standardized dose of pyrogen was 1.0° on the first day as compared to 0.6° on the thirteenth day. It would thus appear that the decline in response to a standard dose of pyrogen which has been reported by previous investigators (19) may, at least in part, be due to the fact that fluctuations of body temperature due to handling and excitement become less pronounced as the animals become accustomed to the experimental procedure. This factor is recognized in the U. S. Pharmacopœia Assay which stipulates that new rabbits must undergo a pretest before the performance of an actual assay and that such a mock test must be repeated when an animal has not been used for more than two weeks. However, our experiments lead us to believe that it requires considerably more than one or two days before an animal becomes completely accustomed to the experimental procedure.

In the light of these findings the temperature-lowering effect of saline and certain bacterial pyrogens reported by Probey and Pittman (9) might be open to a different interpretation. In the case of saline, the lowering of the temperature would simply constitute the normal fall of body temperature in undisturbed rabbits during the first hour of observation. In the case of pyrogens, it might be due to a one- to two-hour lag between the time of injection and the beginning of the pyrogenic effect. The lowering of the temperature observed during the first hour might again, as in the case of saline, reflect the normal temperature of an undisturbed animal which has not yet begun to respond to the injected pyrogen.

In addition to the day-to-day decline in the response to handling there is an immediate temperture rise of 0.2° to 0.3° following the insertion of thermometers, which decreases as the animals become more used to the procedure. This reaction is, of course, completely avoided by the use of stationary rectal thermocouples.

Our experiments with continuous recording show that the temperature rise following handling of a rabbit reaches a maximum within one and one-half

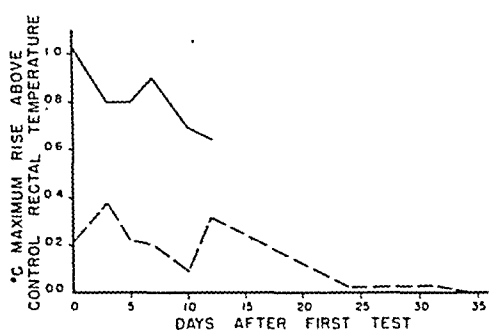

Fig. 6.—Curves showing the decrease in temperature response as additional tests were performed in two groups of rabbits not used prior to the first test in this experiment. In one group of 17 rabbits six tests were conducted in which 4 minimum pyrogenic doses were injected intravenously (solid line). In a different group of 13 rabbits nine tests were performed in the usual manner except that no injections were made (broken line).

to two minutes and declines during the following ten to thirty minutes; on the other hand, it takes between sixty to ninety minutes before the lowest "basal" temperature of an animal has become established.

These observations suggest that in order to avoid readings on a declining slope of the temperature curve one should wait sixty to ninety minutes after the first removal of the animal from its cage before starting an experiment; further, if the (relatively minor) temperature rises due to each insertion of a thermometer are to be avoided, a stationary rectal temperature measuring device should be used.

The technique just described results in a generally lower average starting temperature and a relatively greater sensitivity to thermogenic stimuli. However, since the absolute temperature rise after injection of a standard dose of pyrogen is greater at lower and smaller at higher starting temperature levels, it is obvious that the permissible limit of maximum temperature rises after injection of a test sample should be adjusted to the starting temperature. Figure 3 shows that such an adjustment can easily be made.

These modifications in technique render pyrogen tests more sensitive and convenient; they do not, however, improve the quantitative aspects of the assay. In order to accomplish this purpose it is necessary to follow the principles used in other quantitative bioassay procedures, namely, (*a*) comparison of the unknown sample with a known standard and (*b*) use of an average group response as the basis for biometrical evaluation. With these modifications, the presently used technique can readily be made more quantitative.

In order to estimate the pyrogen content of an unknown sample, it would seem advisable to compare the responses following the intravenous injection of, for example, three 4-fold dilutions of the unknown with 1, 4 and 16 minimum pyrogenic doses of the standard. At least 3 to 5 rabbits should be used on each dose level and the average of the individual temperature rises taken as the criterion of response. The directions in the U. S. P. procedure may generally be followed, except that the control temperature should be taken not sooner than one hour after removal of the animals from the cages. The minimum pyrogenic doses equivalent to the responses with the unknown can be estimated from the dosage response curve drawn for the three responses with the standard; or biometrical procedures similar to those described recently by Knudsen and Randall (18) may be applied. In line with accepted bioassay procedure a standard curve must be obtained for each assay.

The selection of a standard pyrogenic substance is not easy. Although it is relatively simple to prepare a concentrate of high pyrogenicity, and such a concentrate, if kept refrigerated or lyophilized and in sealed ampuls, is stable for at least one year (in contrast to some chemically pure pyrogens which are highly unstable), it must be kept in mind that bacterial pyrogens of different origin vary widely in their pharmacological and toxicological properties. This factor has been recognized in the various collaborative studies initiated by the U. S. Pharmacopœia Revision Committee which selected a pyrogen standard consisting of a mixture of five different pyrogens of bacterial origin.

Unfortunately, the term "pyrogen" is often used rather loosely as if it were to designate a single, pharmacologically well-defined agent. Indeed, many investigators and clinicians abroad are still reluctant to pay much attention to pyrogenic contaminants and the U. S. Pharmacopœia is the only one which officially requires pyrogen tests and prescribes a method for this purpose. A thorough chemical, pharmacological, toxicological and clinical study of this entire field would therefore appear in order.

SUMMARY

1. The manipulations concomitant to the insertion of a rectal thermometer in rabbits are likely to cause an immediate rise in body temperature, reaching its peak within sixty to ninety seconds and requiring ten to thirty minutes for its return to normal.

2. The body temperature of rabbits steadily declines on an average of 0.5° during the first sixty to ninety minutes after removal from their cages.

3. The absolute response to a standard dose of pyrogens is greater at lower than at higher body temperatures.

4. The pyrogen test can be made more quantitative if the average temperature response of the group of rabbits is used as basis for evaluations.

5. The data indicate a linear relationship between the average maximum temperature rise and the logarithm of the dose of pyrogen.

6. The conduct of a quantitative test should involve comparison with a reference standard at several dose levels.

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Stable Ascorbic Acid Solution for Parenteral Use*

By JOSEPH L. CIMINERA and PAUL W. WILCOX

The preparation of a stable buffered solution of ascorbic acid suitable for parenteral administration is described. Trisodium phosphate is used to buffer the solution at *p*H 6.0 to 6.5. Manufacture and storage are done in an atmosphere of nitrogen to prevent oxidation. Sterilization is accomplished by heating sealed ampuls for ten minutes at 100°. This is made possible by filling into small-size ampuls (2 cc.) and by incorporating phenol in a concentration of 0.5 per cent.

SINCE ascorbic acid was made available in quantity, many patents concerning the preparation of this chemical in a stable form suitable for parenteral use have issued. The methods described involve the formation of a salt or ester of ascorbic acid in solution and usually include sealing in ampuls under an inert gas with or without the addition of an antioxidant. Some include the preparation of the iron, nickel, cobalt, manganese, cadmium, or zinc salt (1); the magnesium salt (2); the sodium salt in anhydrous alcoholic solution (3); an aqueous solution of the salt of a base of the class consisting of the alkali metals, alkaline-earth metals, ammonia, and the lower alkali-substituted amines, which solution contains a sulfur-containing reducing agent (sulfur dioxide, sulfur-containing acids capable of yielding SO_2, and soluble salts of them) (4); the double calcium salt prepared by the interaction of equimolecular amounts of the calcium salt of quinic acid and the calcium salt of ascorbic acid (5); the quinine and quinidine esters (6); the benzoyl and veratroyl esters (7); the histidine ester (8); the iron, manganese, calcium, bismuth, arsenic, silver, gold, mercury, copper, zinc, aluminum, or tin salt (9); the aliphatic amine esters (10); the ephedrine, amphetamine, or epinephrine ester (11); and the use of thiourea and the methyl and ethyl derivatives of thiourea as antioxidants (12). In addition, the following have appeared on the market at one time or another: sodium ascorbate, monoethanolamine ascorbate, calcium ascorbate, methyl glucamine ascorbate, ascorbic acid crystals, and others.

The above brief review indicates the complexity of the patent picture in this field. Consequently, we have endeavored to develop a stable buffered solution of ascorbic acid that does not infringe any existing U. S. patent, and is acceptable for direct parenteral administration or for addition to intravenous infusions.

To the best of our knowledge, an aqueous solution of ascorbic acid buffered with an alkaline sodium salt has not been described in the U. S. patent literature. After considering several alkaline sodium salts that are available we decided to investigate the possibility of using trisodium phosphate. The use of this compound for buffering ascorbic acid solutions has been described previously (13). However, no details were given for preparing and storing such solutions in a

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* [illegible] 1946, from the Department of Pharma[illegible]ical Research Division, Sharp & [illegible] Pa.

manner that would guarantee prolonged stability of vitamin C activity. It is the purpose of this paper to present full particulars of a successful procedure.

EXPERIMENTAL

A 10% solution of ascorbic acid was titrated with a solution of trisodium phosphate of the same concentration. Since the solution of ascorbic acid began to discolor at *p*H 7.0 and acquired a yellow-brown color above *p*H 8.0, it was decided to buffer the solution at *p*H 6.3, corresponding to the addition $^1/_3$ mole—equivalent of trisodium phosphate. Accordingly a solution of ascorbic acid was prepared as follows:

Per Cc.		
50 mg. + 10%	Ascorbic Acid.....	55 Gm.
60 mg.	Trisodium Phosphate, $Na_3PO_4 \cdot 12H_2O$ (Mallinckrodt Analytical Reagent)....	60 Gm.
0.5%	Phenol, crystals.....	5 Gm.
	Distilled Water, pyrogen-free, *q. s.*.....	1000 cc.

A volume of 900 cc. of freshly distilled pyrogen-free water was heated to boiling. This was saturated with nitrogen while cooling to 30–35°. The ascorbic acid was added and stirring was continued until complete solution occurred. The trisodium phosphate then was added and dissolved. Finally the phenol, previously melted on a water bath, and sufficient freshly distilled pyrogen-free water (saturated with nitrogen) to make 1000 cc. were added. Stirring was continued under nitrogen until solution was complete. The solution was filtered through a sintered glass filter of medium porosity in an atmosphere of nitrogen and then was filled into sterile flame-sealed ampuls of 2-cc. capacity. This was carried out in an atmosphere of nitrogen under pressure and the ampuls were flushed with nitrogen at 5 pounds pressure for at least two seconds *before* and *after* filling. The sealed ampuls were sterilized by immersion in boiling water, allowing the temperature of the water bath to rise *rapidly* to the boiling point again, and maintaining this temperature for *exactly* ten minutes. The ampuls were immediately removed, and quickly cooled by immersion in running water at 20–25°.

Particular care was taken to exclude air and contact with metals during preparation. The ampuls were stored in a cool place protected from light.

The effectiveness of the sterilization procedure was checked by contaminating the solution with *B. subtilis* spores, and testing for sterility after the heat treatment. Ten thousand spores were added to each cubic centimeter of solution. The *B. subtilis* used was a particularly heat-resistant strain that had been lyophilized on powdered talcum. The concentration of spores in the powder was 2,000,000 per gram. Suitable control experiments proved that the organisms in this powder were viable.

All assays were conducted by the titration method, using 2,6-dichlorophenol-indophenol as suggested by Tillmans (14).

RESULTS

Under these conditions, our ampul solution was sterile after the heat treatment and the *p*H was not altered. Manipulation during preparation resulted in a slight loss of ascorbic acid. However, no further loss was encountered after storage for twelve months in the dark at room temperature. The actual figures were as follows:

	Assay	*p*H
Theoretical	55.0 mg./cc.	6.3
Initial assay before sterilization	50.9 mg./cc.	6.27
Assay after sterilization	51.3 mg./cc.	6.25
Assay after 12 months' storage in the dark at room temperature	50.1 mg./cc.	6.22

DISCUSSION

Ascorbic acid and its salts are known to deteriorate in solution, especially in the presence of air, traces of metals such as copper and iron, and light (15). The most characteristic property of ascorbic acid is its strong reducing action in solution and its ease of oxidation, a reaction that is catalyzed by some metals, especially copper (16) and silver (17).

Ascorbic acid in crystalline form is quite stable. Some laboratories have, therefore, resorted to marketing the dry chemical in sterile vials. This procedure is both tedious and costly. Unless the ascorbic acid in this form is dissolved in a buffer solution or in a solution of a substance capable of partially neutralizing it before use, the resulting solution will be quite irritating when it is injected subcutaneously or intramuscularly. This is due mainly to the inherently low *p*H of solutions of ascorbic acid in distilled water, and is a substantial reason for the partial neutralization of ascorbic acid by the preparation of salts or esters, such as those heretofore described.

The basic material we have employed for partial neutralization of ascorbic acid is an analytical reagent grade of trisodium phosphate, $Na_3PO_4.12H_2O$. This salt has several advantages over many of the other compounds that have been used. It is of more definite chemical composition and is more readily handled than is sodium hydroxide. Both sodium bicarbonate and sodium carbonate react with ascorbic acid with the evolution of carbon dioxide, which has to be removed by degassing under vacuum (4). The analytical reagent grade of trisodium phosphate is substantially free of copper, silver, and other metals detrimental to the stability of ascorbic acid. Another virtue of this salt is its high buffer capacity, which is far superior to that of the other compounds mentioned.

As discussed above, our solution of ascorbic acid began to discolor at *p*H 7.0 and acquired a yellow-brown color above *p*H 8.0. This seems to support

the findings of others that solutions of ascorbic acid are oxidized rapidly upon exposure to light and air, particularly when the *p*H approaches 7.0 (18).

In our preparation it was found desirable to buffer the solution to *p*H 6.3 (6.0–6.5) by the addition of 0.5 mole-equivalent of trisodium phosphate. This *p*H is sufficiently high to prevent discomfort at the sight of injection and yet is well below the critical point where ascorbic acid becomes more easily oxidized.

In order to prevent oxidation of ascorbic acid during manipulation and storage, rigid measures were taken to exclude air. The water used first was boiled to remove dissolved gases and then was saturated with nitrogen during cooling. All subsequent manipulations were carried out under nitrogen. The importance of this step cannot be overemphasized.

The solution may be sterilized by filtration through a sterile all-porcelain candle. All candling procedures, however, entail a rigid aseptic technique to ensure sterility. Wherever possible, it is advantageous to sterilize solutions in sealed ampuls by the use of heat.

At one time it was believed that ascorbic acid was heat-labile, but it is now known that the destruction of the vitamin is an oxidative process. Ascorbic acid can be heated for long periods of time without change, provided all contact with oxygen is excluded (19). Since complete absence of oxygen cannot be assured in the manipulations required for mass production, some oxidation can be expected to occur in solutions that are subjected to autoclaving temperatures or even to the milder heat of tyndallization. The application of two basic principles of sterilization will, however, obviate the need for high temperatures and prolonged periods of heating in order to bring about sterility in solutions. The first is concerned with the size of the container. Underwood has shown that the smaller the container the shorter is the heating period required to bring about sterility of solutions (20). The second principle has to do with the effect of increase in temperature on the germicidal power of phenol. Tilley has shown that there is a considerable increase in this power with an increase in temperature (21). We have applied these two principles to our solution and have found that ten minutes' heating at 100° effectively will sterilize our ampuls provided the size is restricted to 2 cc. or less and provided that phenol in a concentration of 0.5% is present in the solution. No loss of ascorbic acid and no discoloration occurred in our solution when it was subjected to this combined treatment.

The method of sterilization probably could be used for ampuls of larger size, but the period of heating might have to be extended. This could easily be determined by trial.

We have described the preparation of a buffered, stable solution of ascorbic acid containing 50 mg. of ascorbic acid in each cc. of solution. Concentrations up to 200 mg./cc. may be prepared similarly, provided the ratio (by weight) of trisodium phosphate to ascorbic acid is maintained at 12:11.

Our solution was added to intravenous infusions such as normal salt solution, Locke–Ringer's solution, 5% dextrose solution, and human blood plasma without evidence of precipitation. The quantity added represented 100 mg. of ascorbic acid per liter of intravenous infusion.

SUMMARY AND CONCLUSIONS

1. A method for preparing a stable, buffered solution of ascorbic acid is presented.

2. Stability is maintained by adherence to rigid anaerobic conditions by the use of nitrogen throughout manufacture and storage.

3. Solutions containing 50 to 200 mg. of ascorbic acid per cubic centimeter may be buffered to *p*H 6.0–6.5 with an analytical reagent grade of trisodium phosphate, $Na_3PO_4 \cdot 12H_2O$. This is accomplished by using 12 parts by weight of trisodium phosphate to 11 parts of ascorbic acid.

4. Sterilization is accomplished by including phenol in a concentration of 0.5 per cent and by heating the sealed ampuls (2 cc.) at 100° for ten minutes and then cooling.

5. A solution so prepared was found to be stable for at least twelve months at room temperature in the dark.

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Effect of the Anesthetic and the Rate of Injection of Digitalis upon Its Lethal Dose in Cats*,†

By HARALD G. O. HOLCK, KAZUO K. KIMURA, and BARBARA BARTELS‡

In parallel experiments six groups of 12 cats each were anesthetized with urethane and a like number with ether for injection with different dilutions of digitalis according to the U. S. P. XII method. The curves for urethane and for ether were parallel, indicating that the two methods of anesthesia gave results of about the same precision. The smallest lethal dose of digitalis has been calculated to result with 1.76 per cent of tincture of digitalis with either anesthetic. The lethal dose of digitalis was 6.5 per cent greater with urethane than with ether anesthesia; the difference is statistically significant. The slope of the line shows that an error of about 8 per cent exists between the extremes of the 13 to 19 injections permitted by the Pharmacopœia, due to change in concentration of tincture alone.

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and their interaction or error as in a randomized block experiment. The variation between doses was divided into a linear term, a quadratic term, and a remainder. If the lethal dose of digitalis depended upon the concentration of the solution of tincture, it should be possible to express the relation between the two as a curve with linear and quadratic terms. One or both terms should be highly significant. If this curve were substantially a straight line over the entire range of concentrations, the quadratic term should not be significant in comparison with the remainder or error. The mean square for the remaining 3 degrees of freedom between doses should approximate the interaction of doses by groups or error. In both cases the quadratic term was larger than the error although not significantly so and the error terms agreed with each other well within expectation.

For these reasons the two series of experiments have been combined into a single analysis of variance as shown in Table II.

They were related similarly to the log-concentration of the injection fluid, a relation which departed but little from a straight line. The design of the experiment in groups increased its precision appreciably.

The mean response at each rate of injection was then plotted on cross-section paper as in Fig. 1. In both cases the lowest concentration of injection fluid gave a higher lethal dose than the next larger concentration. The two series of points paralleled one another as would be expected from the analysis of variance. The data were then examined in respect to two questions.

The first was to determine the concentration of injection fluid which would lead to the minimum dose since this seemed to be bracketed by the observed range. This can be determined from the ratio of the regression coefficients in fitting a quadratic to the three or four observations covering the range which includes the minimal value. In consequence a new curve was computed by least squares from the totals for both urethane and ether anesthesia at the four lowest concentrations of digitalis. The equation was of the form

$$Y = a' + b_1x + b_2x^2,$$

where x is the log-concentration of digitalis. The ratio of b_1 to $2b_2$ gives the value of x at which Y is a minimum. In terms of the log-concentration of digitalis solution, this proved to be

$$x_{min} = 0.246 \pm 0.142$$

TABLE II.—ANALYSIS OF VARIANCE OF THE INDIVIDUAL LOG-DOSES OF DIGITALIS

Term	Degrees of Freedom	Mean Square or Variance	Variance Ratio or F	
Urethane x ether	1	0.026923	...	5.08[a]
Between groups of cats	22	0.005303	1.83[a]	1.00
Combined slope, b_c	1	0.239216	82.46[a]	...
Quadratic, combined results	1	0.009249	3.19	...
Comparing urethane and ether				
In slope	1	0.002857	0.98	...
In quadratic curvature	1	0.000110	0.04	...
Variation of means about separate curves for urethane and ether	6	0.002321	0.80	...
Error:	110	0.002901	1.00	...
In urethane experiments	55	0.003260	...	...
In ether experiments	55	0.002542	...	...

[a] Statistically significant, $P < 0.05$.

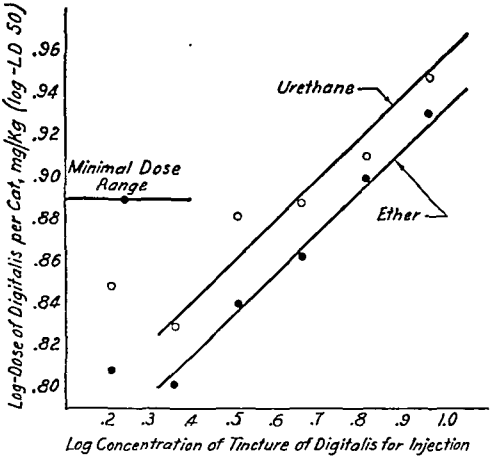

Fig. 1.—A plot of the mean log-doses for data present in Table III.

From the above analysis of variance it is evident that the two anesthetics gave results of about the same precision as judged from the sampling error.

TABLE III.—MEAN LOG-DOSES FOR DATA ANALYZED IN TABLE II AND PLOTTED IN FIG. 1

	Log-Concentration Digitalis	Mean Log-Dose per Cat in Mg./Kg. Urethane	Ether
Plotted points	0.204	0.8489	0.8091
	0.355	0.8275	0.8008
	0.505	0.8816	0.8380
	0.656	0.8888	0.8615
	0.806	0.9098	0.8995
	0.957	0.9469	0.9305
Straight line	0.32	0.8254	0.8005
	1.02	0.9621	0.9372

From the antilog of this result the smallest lethal dose of digitalis in cats was observed when the injection fluid had a concentration of 1.76% of tincture of digitalis. Within limits of one standard deviation this represented 1.27% to 2.45%.. Judging from the above analysis of variance, the two forms of anesthesia did not differ significantly in the concentration of digitalis which gave the smallest dose in mg./Kg. If a dilution of the injection fluid were to be selected so as to obtain the minimal lethal dose per cat, an average of 40 instead of the 15 injections in the U. S. P. XII would be required on the basis of these experiments.

The second point at issue was the relative toxicity of digitalis under ether and urethane anesthesia. This could be measured in two ways, from the relative concentrations of injection fluid and from the relative doses at equal rates of injection. For the first procedure the lowest dose was omitted and the results recomputed from the five higher concentrations. The parallel lines shown in the figure have been drawn with this combined slope $b_c = 0.1953 \pm 0.0595$. The horizontal distance between these two lines indicates the relative toxicity in terms of the concentration of digitalis of the urethane-anesthetized cats as compared with those anesthetized with ether. By this definition the log-ratio of toxicity was $M = 0.1272 \pm 0.0494$. In other words, a 34% greater concentration of digitalis was required to give the same lethal dose in cats that had been anesthetized with ether as in those anesthetized with urethane. The second method was to compare the lethal dose with each anesthetic at corresponding concentrations of injection fluid, representing the first row in the analysis of variance. In terms of the log-toxic dose computed from all records, the mean difference was 0.02735 ± 0.01214. At equal concentrations the lethal dose of digitalis was 6.5% greater with urethane than with ether anesthesia, a statistically significant increase.

Another point worthy of note is the close agreement between the slope established in the present studies, $b_c = 0.195 \pm 0.060$ and the one reported by Bliss and Allmark (1), 0.201 ± 0.053, the latter having been calculated from only 4 concentrations. An error of about 8% could arise in the pharmacopœial digitalis assay if the number of injections were to differ from 13 to 19, between the standard and the unknown or test preparations. On the average, the potency of the unknown should be increased about 1.33% for every injection the unknown is lower than the standard or decreased by about the same amount for every injection it is higher.

In these studies the toxic dose with urethane was only 6.5% greater than those with ether, although a difference of 19% to 20% was reported in two previous studies using several samples of digitalis (2, 3). Since the latter values were based on many cats, a question may be raised as to the source of the difference. In each case it apparently held over a wide range of fatal dosage and comparisons were made both with cats receiving the same concentration and with those having about the same survival time. The fact that the administration was continuous rather than intermittent in the earlier studies can hardly explain the difference.

SUMMARY

1. Cats were anesthetized with urethane or ether and injected with different concentrations of digitalis in parallel experiments. The animals were arranged in 24 groups of six, one cat being tested with each of the following concentrations: 1.60, 2.26, 3.20, 4.53, 6.40, and 9.05 per cent of tincture of U. S. P. Reference Digitalis (1942). The data on each cat included its body weight, heart weight, and the lethal dose of drug per Kilogram of cat, using the U. S. P. XII method for assaying digitalis.

2. Although the heart weight provided an additional correction, the gain in accuracy was insufficient to compensate for the greater complexity of the analysis. In the analysis the log-(mg./Kg.) has been used as the measure of response to digitalis in each cat.

3. Curves for urethane and for ether anesthesia relating the logarithm of the lethal dose of digitalis to the log-concentration of injection fluid were parallel. Accordingly, the two experiments were analyzed together, the two methods of anesthesia giving results of about the same precision.

4. With either anesthetic the lowest concentration of injection fluid gave a higher lethal dose than the next larger concentration. The smallest lethal dose of digitalis would be expected with 1.76 per cent of tincture of digitalis, the limits of one standard error being 1.27 and 2.45 per cent. This value did not differ significantly between the two anesthetics.

5. A 34 per cent greater concentration of digitalis was required with the 5 stronger concentrations to give the same lethal dose in cats anesthetized with ether as in those anesthetized with urethane. At equal concentrations of injection fluid, the lethal dose of digitalis was 6.5 per cent greater with urethane than with ether anesthesia, a statistically significant difference.

6. The slope of the line for the 5 stronger concentrations confirmed the value reported by Bliss and Allmark (1) that an error of about 8 per cent in potency could occur due to the effect of concentration, if an assay included the extremes of thirteen and nineteen injections.

7. The error due to inclusion of bladder urine in the body weight was less than 1 per cent in 80 per cent of the cases, and exceeded 2 per cent in only two of the cats.

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United States Pharmacopœial Convention

Sixth Annual Financial Report, Covering the Period from May 1, 1945, to April 30, 1946, and Based Upon the Report of the Auditor

STATEMENT OF INCOME AND EXPENSE FOR THE YEAR ENDED APRIL 30, 1946

Income			
Sale of Pharmacopœias:			
Collections		$34,904.75	
Add: Increase in Accounts Receivable			
April 30, 1946	$ 5,411.00		
April 30, 1945	4,868.25	542.75	$35,447.50
Sale of Reference Standards, A. M. A. Articles, etc.:			
Collections		$ 6,188.94	
Add: Increase in Accounts Receivable			
April 30, 1946	$ 916.50		
April 30, 1945	559.50	357.00	
		$ 6,545.94	
Less: Decrease in Collections Pending Remittance to the Treasurer			
April 30, 1945	$ 825.61		
April 30, 1946	719.25	106.36	6,439.58
Interest on Investments:			
Collections		$ 2,431.25	
Add: Increase in Accrued Interest Receivable			
April 30, 1946	$ 830.74		
April 30, 1945	742.19	88.55	
		$ 2,519.80	
Less:			
Amortization of Bond Premiums	$ 157.29		
Purchase of Accured Interest Receivable	18.36	175.65	2,344.15
Miscellaneous Income:			
Collections		$ 180.91	
Add: Net Adjustment of Furnishings and Equipment Account and Reserve		64.75	245.66
Total Income			$44,476.89

Less:				
Expenses				
Publication and Sales Expense		$11,207.25		
Add: Decrease in Inventory				
April 30, 1945	$6,630.34			
April 30, 1946	4,014.59	2,615.75	$13,823.00	
Administration			3,636.92	
Convention			16.99	
Revision		$24,332.98		
Add: Decrease in Inventory Reference Standards				
April 30, 1945	$2,796.18			
April 30, 1946	2,073.02	723.16	25,056.14	
Research			6,177.73	
Stationery and Supplies Inventory Adjustment			111.57	
Provision for Depreciation:				
Building		$ 241.23		
Furnishings and Equipment		202.49	443.72	$49,266.07
Excess of Expense over Income				
For the Year Ended April 30, 1946				$ 4,789.18

STATEMENT OF CASH RECEIPTS AND DISBURSEMENTS (GENERAL ACCOUNT) FOR THE YEAR ENDED APRIL 30, 1946

Cash Receipts and Disbursements		
Cash on Deposit, April 30, 1945		$ 67,368.65
Add:		
Receipts		
Sale of Pharmacopœias	$34,904.75	
Sale of Reference Standards, A. M. A. Articles, etc.	6,188.94	
Sale of Securities	15,000.00	
Interest on Investments	2,431.25	
Use of Text by Others	15.00	
Miscellaneous	165.91	58,705.85
		$126,074.50

Less Disbursements	Publications and Sales	Administration	Convention	Revision	Research	Total
Printing and Binding	$11,142.44	.....	.....	.....	.....	$11,142.44
Honoraria	.....	$1,650.00	.....	$10,175.00	.....	11,825.00
Clerical	.....	1,101.06	5.03	6,309.46	144.80	7,560.35
Meetings	.....	320.12	.....	1,522.59	1,052.51	2,895.22
Supplies	.....	45.85	9.00	968.18	353.62	1,376.65
Postage and Telegrams	.....	95.60	2.04	955.59	28.23	1,081.46
Compensation	.....	.....	.....	.....	4,550.04	4,550.04
Headquarters	.....	.....	.....	1,303.39	.....	1,303.39
General	64.81	424.29	0.92	3,098.77	48.53	3,637.32
	$11,207.25	$3,636.92	$16.99	$24,332.98	$6,177.73	$45,371.87

Add:		
Purchase of Securities	$15,000.00	
Purchase of Accrued Interest Receivable	18.36	
Purchase of Furnishings and Equipment	1,406.50	
Building Improvements	1,863.53	$63,660.26
Cash on Deposit, April 30, 1946		$62,414.24

A MORE DETAILED AND SUPPLEMENTARY STATEMENT ON THE REVISION AND RESEARCH EXPENSES

REVISION—MEETINGS

Chairman's Conferences	$ 1,159.11
Hearing, U. S. P. XIII	363.48
	$ 1,522.59

REVISION—SUPPLIES

Stationery	$ 224.40
Mimeograph Supplies	396.00
Miscellaneous Office Expenses	347.78
	$ 968.18

REVISION—SALARIES

Revision Chairman, E. Fullerton Cook	$ 7,375.00
Executive Assistant, Adley B. Nichols	2,800.00
	$10,175.00

REVISION—GENERAL

Reference Standards—Maintenance and Renewals	$ 542.36
Reference Standards—New	2,226.26
Pharmacopœia & Physician Articles—Spanish	182.04
Inter-Society Color Council	25.00
Miscellaneous Expenses	123.11
	$ 3,098.77

BUILDING

Moving	$ 252.84
Maintenance—General and Janitorial	1,050.55
	$ 1,303.39

RESEARCH

Subcommittee	Meetings, Supplies, Postage, Clerical and General	Technical Assistance	
1. Scope	$ 15.70	Dr. Frederick K. Bell, Asst. to Dr. Krantz	$2,500.04
5. Botany & Pharmacognosy	30.39	Kenneth Waters, Asst. to Dr. Beal	$1,800.00
7. Inorganic Chemicals	163.57	Laboratory Assistant—Running Assays and Caring for Animals	$ 250.00
8. Organic Chemicals	4.26		
10. Volatile Oils	30.00		
13. Ointments	318.19		
Penicillin Study	6.40		
Anti-anemia Products Board	195.73		
Endocrine Products Board	737.73		
Sterile Products Board	61.80		
Vitamin Board	31.90		$4,550.04
Digitalis Study	32.02		1,627.69
	$1,627.69		$6,177.73

Total .....

The Chemical Assay of Penicillin*,†

By MERLIN MUNDELL, HENRY FISCHBACH, and THOMAS E. EBLE

A number of methods for the chemical determination of penicillin have been studied. Two methods, namely, the penicillinase procedure and the method based on the iodometric titration, have been compared with the bio-assay on a large number of samples of penicillin. Data are reported which indicate that the results of either of the chemical methods are more accurate and reproducible than the results of the bioassay. Due to certain technical difficulties inherent in the penicillinase procedure, the authors prefer the iodometric titration to all other methods studied.

FOUR ANTIBIOTICS of the penicillin group have been isolated from commercial products and all have the empirical formula $C_9H_{11}O_4SN_2 \cdot R$ (1). In penicillin F, R is Δ^2 pentenyl ($—CH_2 \cdot CH = CH \cdot CH_2 \cdot CH_3$); R in penicillin G is the benzyl group ($—CH_2 \cdot C_6H_5$); in penicillin X, R is the *p*-hydroxy benzyl group ($—CH_2—C_6H_4OH$); and in penicillin K, R is the n-heptyl group ($—CH_2 \cdot CH_2 \cdot CH_2 \cdot CH_2 \cdot CH_2 \cdot CH_2 \cdot CH_3$).

Penicillin prepared commercially may contain any mixture of the four types of penicillin but most preparations are believed to contain a mixture of penicillins G and K predominately.

These penicillins are known to differ in their activity against certain organisms. For use as a standard, the activity of pure penicillin G against *Staphylococcus aureus in vitro* has arbitrarily been set at 1667 units per milligram. Under similar conditions, penicillins K and X have been shown to have activities of approximately 2300, and 900 units per milligram, respectively, while penicillin F has a potency which has been variously reported to be 1400 to 1900 units per milligram.

The activities *in vivo* experiments do not parallel those determined *in vitro*. Welch, *et al.* (2) have reported that penicillin X is more effective than commercial penicillin in the treatment of gonorrhea on a unit basis. They also report that preliminary studies indicate that penicillin X is three to five times more effective than commercial penicillin in protecting mice against 10,000 lethal doses of pneumococcus. In view of this variation in activity, the ideal method of assay for penicillin would be one which would determine the amount of each of the penicillins present in the preparation. While methods for that purpose have been proposed, they are as yet too time-consuming to be feasible as routine control procedures. Until a simple procedure has been devised for the determination of the amount of each of the various types of penicillin in a mixture, the control of the production of this material should be based on the method of assay which gives the greatest degree of accuracy and the most uniformly reproducible results.

A cup-plate procedure for the assay of penicillin was first proposed by Abraham, *et al.* (3), and was based on the activity of penicillin *in vitro* against *Staph. aureus*. The authors pointed out that, with the use of three plates, the inherent error was probably not greater than ±25%. This method has undergone numerous refinements with the view of increasing the accuracy of the procedure. One modification of this method was adopted as the official method for the assay of penicillin and is described in the Federal Register for September 8, 1945, page 11478. Further study led to the modification of this assay (Federal Register, June 18, 1946, page 3080). Both of these assay techniques have been used in the studies reported herein.

Several chemical methods have been proposed for the assay of penicillin. Of these the following have been investigated in this laboratory: The colorimetric method proposed by Scudi (4), the penicillinase method (5), the alkali inactivation and the H_2O_2

* Received Oct. 28, 1946, from the Chemical Section of the Medical Division, Food and Drug Administration, Federal Security Agency, Washington, D. C.

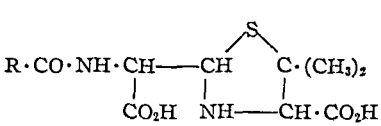
† A portion of this material has been presented by Mr. Eble in partial fulfillment of the requirements for the degree of Master of Science in the Department of Biochemistry, Georgetown University.

inactivation methods proposed by the Chas. Pfizer and Company laboratories (6), the spectrophotometric method proposed by Herriott (7), and the iodometric method proposed by the Squibb Institute for Medical Research (8). The penicillinase method and the iodometric method with some modification seem to give the most consistent results when applied to commercial penicillin products from all manufacturers. Other proposed methods as yet not studied in this laboratory are a colorimetric method proposed by Bristol Laboratories, Inc. (9), and the ninhydrin method (10). This paper is concerned with the results obtained by the penicillinase and the iodometric methods as compared with the results of the two officially adopted procedures referred to above.

EXPERIMENTAL

The Penicillinase Method.—The penicillinase method was developed by Murtaugh and Levy (5). This method is based on the observation of Foster (11) that acid is produced when penicillin solutions are inactivated by penicillinase. Because buffers in the solution interfere with the precise determination of the end point, a highly purified penicillinase preparation is needed. McLuarrie, Liebman, Kluener, and Venosa (12) reported the preparation of such an enzyme which is suitable for use in this assay.

The product of the inactivation of the penicillin is presumed to be penicilloic acid which has the formula:



where R is one of the characterizing groups previously listed. The carboxyl group attached to the thiozolidine ring is identical with the acidic group of penicillin. The other carboxyl, produced by the hydrolysis of the penicillin molecule, is the one measured in the titration. The hydrolysis takes place at the same point in the molecule in all four types of penicillin. The penicillinase method will therefore give the same value for equimolecular quantities of the four penicillins. This has been verified by assaying weighed samples of the crystalline penicillins which were available and results are shown in Table I.

Ten weighed samples of a uniform mixture of sodium sulfate and sodium penicillin G having a potency of 370 units/mg. were dissolved in water and diluted to 25 ml. Aliquots of each of these solutions were taken for duplicate cup-plate and penicillinase assays. The cup-plate assays reported in Table II were obtained by use of the assay described in the Federal Register for September 8, 1945.

TABLE I.—RECOVERY OF CRYSTALLINE PENICILLINS BY THE PENICILLINASE ASSAYS[a]

Sample	Wt. Assayed, Mg.	Wt. Recovered, Mg.	% Recovery
Sodium Penicillin G	5.82	5.77	99.1
	3.31	3.35	101.2
	9.38	9.26	98.7
	7.57	7.53	99.6
Sodium Penicillin F, 175-E-ANWF	4.54	4.83	106.3
	5.68	5.90	103.9
	8.60[b]	8.08	94.0
Sodium Penicillin X, NRRL 1625-7A	6.68	6.27	93.9
	6.68	6.24	93.3
	7.34[b]	6.64	90.5
New Penicillin from culture X1612 (calculated as K)	4.24	4.16	98.1
	5.30	5.04	95.1

[a] The penicillinase used in this work was contributed by the Schenley Research Institute.
[b] Assays made 8 months later than the other assays reported.

TABLE II.—COMPARISON OF THE PENICILLINASE ASSAY AND THE CUP-PLATE ASSAY USING A SAMPLE OF SODIUM PENICILLIN G DILUTED WITH SODIUM SULFATE

Mg. of Mixture Titrated	Units of Penicillin Found[a]	Units/Mg. by Penicillinase Assay[a]	Units/Mg. by Bioassay
36.8	13,450	365	396
	13,450	365	366
38.0	13,900	366	300
	13,750	362	329
38.4	14,200	370	370
	14,200	370	374
39.8	14,600	367	381
	14,600	367	366
36.8	13,700	372	426
	13,850	376	322
40.2	15,350	382	337
	15,050	374	411
42.8	15,350	359	366
	16,000	375	374
41.7	15,200	365	340
	15,350	368	340
37.9	13,750	363	359
	14,200	375	396
38.2	14,100	369	315
	13,850	363	355
	Av.	369	361
Standard Deviation		5.5	31.6
Standard Deviation %		1.5	8.8

[a] Calculated on the basis of 1667 units/mg.

This data shows that for sodium penicillin G the penicillinase and the cup-plate methods give essentially the same values when a sufficiently large number of cup-plate assays are made. The penicillinase method is subject to less variation than the bioassay.

The Iodometric Method.—Although the penicillinase method is well suited for the determination of penicillin in ampuls, a cumbersome extraction is required prior to the inactivation treatment when buffered tablets are encountered. In addition analyses on ointments or troches of low penicillin content are somewhat awkward.

The iodometric method proposed in this paper stems in part from the observations of the Eli Lilly Laboratories (13) and in part from an iodometric procedure suggested by the Squibb Institute of Medical Research (8). The former concluded that sodium penicillin as such is not oxidized by iodine at any *p*H; that the iodine oxidation of sodium penicilloate G or the esters of penicilloate G is probably limited to 9.0 equivalents of iodine per mole. A consumption of 8.5–8.9 equivalents was found by actual experimentation. The latter group, in agreement with the results of the Lilly Laboratories, decided that 2.52 ml. of 0.01 *N* I_2 solution was consumed by the alkaline inactivation product of one milligram of crystalline sodium penicillin G and suggested the conditions for assay.

After consideration of the above information, it was apparent that several time-rate and recovery studies were necessary for the establishing of a fixed procedure. These were carried out at a room temperature of approximately 23°.

Rate of Alkaline Inactivation.—Four-milliliter aliquots of a standard solution of crystalline sodium penicillin G (0.6037 mg./ml.) were added to each of six iodine flasks and 4 ml. of 1.0 *N* NaOH added. Inactivation was allowed to proceed for varying periods of time subsequent to which 4 ml. of 1.1 *N* HCl were added to each flask along with 10 ml. of 0.01 *N* I_2 solution and each permitted to stand for thirty minutes prior to titration with 0.01 *N* $Na_2S_2O_3$.

Rate of Iodine Consumption.—Four-milliliter aliquots of a standard solution of crystalline sodium penicillin G (0.5849 mg./ml.) were added to each of eight iodine flasks and 4 ml. of 1.0 *N* NaOH added and the solution allowed to stand for fifteen minutes. Four milliliters of 1.1 *N* HCl and 10 ml. of 0.01 *N* I_2 solution were then added and time for I_2 consumption was varied from five to forty minutes.

TABLE III.—RATE OF ALKALINE INACTIVATION

No.	No. Min. for Alkaline Inactivation	Ml. 0.01012 *N* I_2 Consumed[a]	% Recovery
1	5	5.85	97.3
2	10	6.00	99.7
3	15	6.05	100.5
4	20	6.05	100.5
5	25	5.95	99.0
6	30	6.05	99.7

[a] The titration of the blank was 9.59 ml. 0.01012 *N* I_2.

It is apparent from the above data that ten to fifteen minutes affords complete inactivation under the prescribed conditions.

TABLE IV.—RATE OF IODINE CONSUMPTION

No.	Time in Minutes for I_2 Consumption	Ml. 0.01012 *N* I_2 Consumed	% Recovered
1	5	5.75	98.3
2	10	5.77	98.6
3	15	5.82	99.4
4	20	5.80	99.1
5	25	5.82	99.4
6	30	5.79	99.0
7	35	5.80	99.0
8	40	5.82	99.4

It appears from Table IV that the reaction between the inactivation product of penicillin and iodine is very rapid, being complete in fifteen minutes.

Finally, it was desirable to ascertain the minimum excess iodine required during the reaction for optimum results. For this purpose 3-ml. aliquots of crystalline penicillin G (0.9004 mg./ml.) were added to each of 11 iodine flasks, inactivated with 3 ml. of 1.0 *N* NaOH for fifteen minutes, 3 ml. of the hydrochloric acid solution, and varying amounts of iodine added. A blank titration was made for each determination. The data are presented in Table V.

Examination of the tabulation below reveals the need of a 25% minimum excess of available iodine in order to achieve a 98% recovery. The

TABLE V.—DETERMINATION OF THE MINIMUM EXCESS IODINE REQUIRED

Exptl. No.	Ml. 0.00970 *N* I_2	Ml. 0.01014 *N* $Na_2S_2O_3$ Blank	Titer After Inactivation, 0.01014 *N* $Na_2S_2O_3$	Ml. I_2 (0.00970 *N*) Consumed	Mg./ml.	% Recovery
1	6.0	5.65	0.0	>5.91	...	...
2	6.5	6.10	0.02	6.36	0.815	90.6
3	7.0	6.70	0.28	6.71	0.861	95.7
4	7.5	7.15	0.71	6.73	0.864	95.9
5	8.0	7.64	1.15	6.78	0.872	97.0
6	9.0	8.64	2.05	6.89	0.884	98.2
7	10.0	9.60	2.98	6.92	0.888	98.6
8	12.5	11.90	5.22	6.98	0.896	99.5
9	15.0	14.61	7.87	7.06	0.906	100.3
10	20.0	19.71	12.66	7.22	0.927	102.6
11	30.0	29.39	22.66	7.04	0.903	100.1

following method, based on these observations, was developed for assaying ampuls of penicillin.

Reagents:

1. 0.01 *N* Iodine Solution
2. *0.01 N Sodium Thiosulfate*
3. 1 *N* Sodium Hydroxide
4. 1.1 *N* Hydrochloric Acid
5. 1% Starch Solution
6. C. P. Carbon Tetrachloride

Method.—Place 5 ml. aliquots of a solution containing penicillin in a concentration of about 0.6 mg./ml. (1000 units/ml.), in each of two iodine flasks. To one add an equal quantity of 1 *N* NaOH and permit to stand at room temperature for fifteen minutes, and then add 5 ml. of the 1 *N* HCl and 15 ml. of 0.01 *N* I_2. After fifteen minutes titrate the excess iodine with 0.01 *N* sodium thiosulfate, approaching the endpoint with an occasional drop of 1% starch solution as an indicator and completing the titration after the addition of about 5 ml. CCl_4. The latter is particularly advantageous for colored solutions. To the second flask add 15 ml. of the 0.01 *N* I_2 solution and titrate immediately for the "blank determination." Difference in titers ÷ 2.52 = mg. penicillin.

It was known that iodometric titrations are more reproducible when carried out at a low *p*H level (*p*H 2 to 3). Advantage was taken of this fact by using a solution of hydrochloric acid which was approximately 0.1 of an equivalent stronger than the alkali used for inactivation. However, for the blank determination, no adjustment of the *p*H was attempted since this would result in the formation of some degradation products which in turn would react with iodine and yield a correspondingly low value for penicillin potency. It should be noted that among the required reagents only the normality of the sodium thiosulfate solution is critical.

The iodometric assays of various concentrations of different batches of crystalline sodium penicillin G are presented in Table VI.

TABLE VI.—ASSAY OF DIFFERENT BATCHES OF CRYSTALLINE SODIUM PENICILLIN G

No.	Concentrations Used, Mg./Ml.	% Recovery	Ml. 0.01 *N* I_2, Mg.
1	2.66	101.2	2.55
2	0.6004	99.0	2.49
3	5.024	100.3	2.53
4	0.6016	99.9	2.52
5	2.999	101.6	2.56
6	3.001	101.0	2.54
7	2.999	100.8	2.54

The average of the above data indicates that 2.53 ml. of 0.01 *N* iodine solution corresponds to one milligram of sodium penicillin G. This is in good agreement with the 2.52 average reported by the Squibb group. The latter value is used for the basis of the calculations in this report.

A few samples purported to be salts of penicillins F, X, and K were received by this laboratory and previously described under the penicillinase method. These were subjected to the iodometric assay and preliminary results indicate that a similar number of equivalents of iodine react with equimolecular quantities of the respective penicilloates resulting from alkaline inactivation. Although the Squibb group suggested that penicillin X might give abnormally high values, the results in this laboratory do not bear out this opinion.

For the sake of brevity, Table VII exhibits only a few of the typical comparative data obtained for commercial vials of penicillin salts utilizing the Iodometric, the Penicillinase, and the Bioassay methods on the same ampul. The bioassay technique used was that described in the Federal Register for September 8, 1945. Each capital letter represents a different commercial source.

TABLE VII.—COMPARATIVE DATA ON COMMERCIAL VIALS

Manufacturer	% Recovery Based on Manufacturer's Claim Penicillinase[a]	Iodometric[a]	Bioassay
A	121	126	134
	110	109	111
	104	104	100
B	113	113	118
	94	95	94
	93	91	96
C	109	102	110
	113	114	109
	116	117	117
D	99	99	90
	106	107	115
	109	108	95
E	101	99	111
	97	95	106
	96	93	109
F	124	123	110
	107	107	119
	107	110	125
G	103	102	113
	103	101	98
	106	101	98
H	104	104	122
	103	103	108
	104	102	117
I	98	99	105
	94	90	93
	97	100	100
J	92	93	90
	95	99	99

[a] Calculated on the basis of 1667 units/mg.

The above results exhibit good agreement between the chemical methods. The recovery data in each case are based on the claims of the respective manufacturer.

The adoption of an improved bioassay technique (Federal Register, June 18, 1946) made a comparison of the results of this method and of the chemical methods desirable. For this purpose thirty samples of crystalline penicillin G, accurately weighed and varying between 25.5 mg. and 34.5 mg., were diluted separately to 50 cc. One analyst made duplicate penicillinase assays using 10-ml. aliquots of each solution; another analyst made duplicate iodometric assays using 5-ml. aliquots; and the Control

TABLE VIII.—COMPARATIVE ASSAYS OF CRYSTALLINE PENICILLIN G

	Weight of Penicillin, Mg.	% Recoveries Penicillinase	Iodometric	Bioassay
(1)A	32.58	102.4	97.3	99.9
B		101.7	97.5	94.8
(2)A	28.13	102.9	99.5	95.8
B		97.9	99.4	94.2
(3)A	30.26	101.7	97.7	95.2
B		101.3	97.3	98.1
(4)A	26.85	104.9	98.0	93.8
B		103.7	97.7	100.5
(5)A	32.58	102.3	97.4	92.2
B		99.9	97.4	97.4
(6)A	33.47	100.5	99.3	97.7
B		97.8	99.8	102.2
(7)A	29.97	99.7	98.8	96.5
B		99.4	100.0	96.0
(8)A	27.01	99.6	99.2	101.0
B		99.6	99.5	98.0
(9)A	31.05	97.9	97.4	99.0
B		99.5	99.1	93.7
(10)A	30.00	96.2	100.3	99.0
B		98.3	100.7	99.0
(11)A	34.18	99.8	100.0	92.1
B		100.4	100.2	92.1
(12)A	26.23	100.4	100.3	106.3
B		102.2	100.9	105.2
(13)A	25.81	99.8	99.1	98.8
B		100.3	99.6	98.8
(14)A	33.96	100.3	99.9	94.8
B		100.1	100.8	94.5
(15)A	32.07	98.6	99.4	93.5
B		99.2	100.2	92.6
(16)A	30.00	100.2	101.1	98.0
B		99.2	100.8	98.0
(17)A	27.56	100.0	101.7	103.4
B		100.7	101.4	103.4
(18)A	25.95	98.8	101.5	111.0
B		99.0	101.7	109.8
(19)A	25.94	99.6	101.5	104.1
B		99.8	100.0	105.2
(20)A	34.33	98.8	98.5	90.0
B		98.8	98.9	94.4
(21)A	34.37	99.9	100.0	99.5
B		99.5	100.1	99.5
(22)A	34.36	100.7	100.6	97.8
B		99.0	100.7	96.0
(23)A	25.95	99.1	100.5	104.0
B		99.6	100.8	91.3
(24)A	25.95	98.3	99.3	102.9
B		99.0	99.2	105.2
(25)A	30.00	99.1	100.6	98.5
B		98.4	100.4	97.5
(26)A	28.25	97.9	100.8	93.4
B		99.3	100.4	101.9
(27)A	26.46	100.7	100.9	97.5
B		100.3	100.1	95.2
(28)A	32.89	97.7	100.4	96.7
B		97.7	100.0	94.8
(29)A	32.16	98.9	101.9	96.1
B		99.0	99.8	95.6
(30)A	27.10	100.6	101.3	98.5
B		99.8	100.8	100.7
	Av.	99.80	99.82	98.20
Standard Deviation % from the mean		1.5	1.2	4.4
Standard Deviation % from the known weight		1.5	1.2	4.8

Laboratory of the Division of Penicillin Control & Immunology made duplicate assays by the bioassay method referred to above on aliquots of the solutions. The analysts were not told the exact weight of material in the sample until their results were completed. The last ten solutions from samples 26 to 30 were marked so that the analyst did not know which pair were duplicates. The results of this study are tabulated in Table VIII.

In the iodometric titration of samples 1 to 5, inclusive, 10 ml. of 0.01 *N* iodine (representing an excess of about 25%) were used. In samples 6 to 30, inclusive, 15 cc. of iodine solution (representing an excess of about 100%) were used. The improved recoveries due to the use of the larger excess of iodine solution are obvious.

DISCUSSION

The results of the bioassay techniques are determined in units of penicillin, where the solution under investigation is compared with a standard penicillin G solution. Unless the proportion of each of the four types of penicillin in the preparation is known, these results cannot be accurately converted to a weight basis. The results of the chemical methods used in this study are most accurately expressed in milliequivalent weights of penicillin without any reference to the type of penicillin involved. Since the molecular weights of the various known penicillins are of the same order, little error is to be anticipated by converting the results from milliequivalent weights to milligrams by multiplying by the average of the molecular weights of the four penicillins.

Without a knowledge of the proportions of the four penicillins present, however, the results of the chemical assay cannot be accurately translated to the unit basis. Since the results of the bioassay and chemical assays of a preparation of unknown composition cannot be expressed accurately in the same terms, any comparison of the accuracy of the two methods must be made on material of known composition. For this reason the standard deviations of various results were calculated only for those studies in which penicillin G was the only antibiotic present.

As shown in Table II, the standard deviation from the mean for the bioassay described in the Federal Register, September 8, 1945, was found to be 8.8%. Results in

Table VIII show standard deviations of 4.4% from the mean and 4.8% from the known weight when the bioassay described in the Federal Register, June 18, 1946, was used. The same tabulations show a standard deviation from the mean and from the known weight of 1.5% for results by the penicillinase method and a standard deviation of 1.2% for the results of iodometric titration.

A comparison of the two chemical methods shows that each has certain inherent advantages and disadvantages. The penicillinase method requires the use of special equipment and is difficult to apply to some dosage forms of penicillin. While no detailed study has been reported on the action of penicillinase, it is safe to assume that it, like other enzymes, is highly specific and a method based on its action would be specific for penicillin. The iodometric method has the advantage of requiring no special apparatus or reagents. It has been successfully applied to most dosage forms thus far studied and in all probability can be adopted for use in other dosage forms. It is not specific for penicillin, however, and, in the absence of information on the composition of the sample, might require a supplementary analysis by the penicillinase method or a crystallographic study.

We hope, through a collaborative study with a number of manufacturers of penicillin to extend this comparison of the accuracy and reproducibility of the results by bioassay and by iodometric titration.

SUMMARY

A number of chemical methods for the assay of penicillin have been studied. The results by two of the methods, the penicillinase method and the iodometric titration, have been compared with the results of the bioassay. The standard deviation of the data obtained by chemical assay was $^1/_4$ to $^1/_3$ the standard deviation of the results of the bioassay. Of all of the chemical methods tested, the authors prefer the iodometric titration for the determination of penicillin.

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WHO MAKES IT?

The National Registry of Rare Chemicals, Armour Research Foundation, 33rd, Federal and Dearborn Streets, Chicago, Ill., seeks information on sources of supply for the following chemicals:

2,3-Dihydroxyanthraquinone
1-Phenyl-3-methyl-5-chloropyrazol
Cinchophen amyl ester
933F (2-Piperidinomethyl-1,4-benzodioxan)
883F (2-Diethylaminoethyl-1,4-benzodioxan)
Crystalline trypsin
2-Phenylbenzopyrone
5,7-Dihydroxy-2-phenylbenzopyrone
2,2′-Dihydroxy-5,5′-dibromobenzil
2,2′-Dihydroxy-3,3′,5,5′-Tetrachlorobenzil
d-Camphor
l-Menthol
3-Chlorotetrahydrofuran
1,1,2,3-Tetrachloroisobutane
1,1,1,2,3-Pentachloroisobutane
1,1,3-Trichloro-2-methyl-1-propene
3-Amino-2,4-dimethylbenzoic acid
2-Methyl-1,3-propylene glycol
1,3-Pentanediol
2-Methyl-1,2-butylene glycol
2-Methyl-1,3-butylene glycol
Pulegone
Thujone
3-Hexen-1-ol

Pharmaceutical Applications of Isopropyl Alcohol. I. As a Solvent in Pharmaceutical Manufacturing*,†

By HENRY M. BURLAGE‡ and DORIS BULLARD HAWKINS

Ten galenical preparations official in the U. S. P. XII and the N. F. VII were prepared with ethyl alcohol and with isopropyl alcohol. Tables of comparison of the specific gravity, *p*H, color, odor, taste, changes on standing, and total extractive of the preparations were compiled.

INTRODUCTION

ISOPROPYL ALCOHOL, dimethyl carbinol, secondary propyl alcohol, CH_3—CHOH—CH_3, was first prepared by Berthelot in 1855 by the reaction of propylene with sulfuric acid and subsequent hydrolysis of the sulfuric acid esters (1). In 1862, Friedel prepared the alcohol by the reduction of acetone (2). Kolbe, in the same year, recognized in isopropyl alcohol the first of the class of secondary alcohols (3). From 1893 to 1900, studies on the compound were made by Zouboff (4), Heinrich (5), and Tischtschenko (6). Others followed in 1904 (7), 1906 (8), 1907 (9), 1909 (10), 1910 (11), 1917 (12), 1920 (13), and 1921 (14, 15).

The first use of the compound in cosmetics appears to have been in 1921 (16). In 1922, the alcohol and glycol were used in the preparation of extracts as a substitute for ethanol (17) and in the same year its pharmacology and applications as a solvent and vehicle were studied (18). Bernhardt in 1922 (19) proposed its use to replace ethyl alcohol in similar concentrations as a disinfectant for the hands. In 1923 the solubilities of certain essential oils in this alcohol were reported (20). It was also recommended in 1926 for use on the skin and clothing (21).

In 1927 and 1928 (22), pharmaceutical applications of the alcohol were reported and Schamelhout's investigations (23) suggested that this compound, because of its similar properties, might be used as a substitute for ethanol in the determination of acid numbers, saponification values of fats, oils, and balsams, and in other tests. In 1938, Ducommun (24) showed that in using a mixture of acetone, isopropyl alcohol, and water in preparing extracts of Cinchona, Belladonna, Nux Vomica, Rhubarb, and Gentian, the active principles were more rapidly extracted than with ethyl alcohol. In 1938, it was used as a solvent in place of ethanol in preparing certain dry extracts of the Swiss Pharmacopœia with similar or improved results (25).

In 1943, its lauric, myristic, and palmitic acid esters were suggested for use as carriers in cosmetic creams, medicaments, perfumes, and flavors (26). In 1944, Lehman and his co-workers (27), using secondary propyl alcohol, prepared Tincture of Digitalis of equivalent or greater potency than one made with the official menstruum. In the preceding year its use in hand soaps (28) and in Liniment of Soft Soap and Liniment of Camphor and Soap was recommended (29). Harris, in his studies on Tincture of Iodine, found that isopropyl alcohol as a solvent yields a product equal to the official preparation in iodine content and antispetic action (30).

Isopropyl alcohol possesses a lower surface tension, a greater fat solvent action, a more rapid killing power on many organisms, is less expensive than ethyl alcohol, and is free from taxation. It has been recommended for use in the preparation of extracts where the solvent is evaporated, in the production of tablet granulations, in pharmaceutical preparations for external use, in diagnostic stains, and in addition has been recently accepted for inclusion in the National Formulary. Therefore a study of its value as a menstruum and solvent in a number of official preparations was undertaken.

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As a basis for the selection of the preparations to be studied, ten which ranked high in frequency of use in a prescription survey recently conducted by Burlage (31) were chosen. These included:

Tincture of Nux Vomica U. S. P. XII.
Tincture of Belladonna U. S. P. XII.
Tincture of Stramonium U. S. P. XII.
Tincture of Hyoscyamus U. S. P. XII.
Camphorated Tincture of Opium U. S. P. XII, First Supplement.
Aromatic Fluidextract of Cascara Sagrada U. S. P. XII.
Elixir of Terpin Hydrate N. F. VII.
Elixir of Terpin Hydrate with Codeine N. F. VII.
Compound Tincture of Cardamon U. S. P. XII.
Elixir of Phenobarbital U. S. P. XII, First Supplement.
Elixir of Three Bromides N. F. VII.
Aromatic Spirit of Ammonia U. S. P. XII.
Compound Elixir of Pepsin N. F. VII.
Elixir of Iron, Quinine, and Strychnine N. F. VII.
Elixir of Five Bromides N. F. VII.
Aromatic Elixir U. S. P. XII.
Fluidextract of Cascara Sagrada U. S. P. XII.

All products except Aromatic Elixir were prepared by official procedures. Aromatic Elixir was manufactured by Shiflett's method (32), which the authors have found to be more satisfactory than the pharmacopœial procedure.

In making these preparations it was found that the following made from isopropyl alcohol, filtered more rapidly than those prepared from ethanol: Camphorated Tincture of Opium, Elixir of Terpin Hydrate, Compound Tincture of Cardamom, and Elixir of Three Bromides; and much more rapidly: Aromatic Elixir and Elixir of Five Bromides.

It was also observed that terpin hydrate dissolved very slowly in isopropyl alcohol and readily in ethanol; that phenobarbital was slightly less readily soluble in isopropyl alcohol than in ethyl alcohol.

EXPERIMENTAL

Physical Constants and Properties of the Products

Specific Gravity.—This constant was determined with a Mohr–Westphal balance at 25° and the values are reported in Table I.

***p*H Values.**—These were determined with a Coleman Electrometer and are reported in Table II.

Physical Properties.—The physical properties studied included (*a*) color of the freshly prepared products (Table III), (*b*) color of the products after storage of nine months or more (Table IV), (*c*) odor (Table V), and (*d*) taste (Table VI).

Determination of Total Extractive

The total extractive of the preparations made by maceration and percolation was determined in the following manner:

Measure 100 cc. of the preparation by means of a transfer pipette into a tared glass evaporating dish which has been previously heated to constant weight in the oven. Allow the sample to evaporate and dry in the air until consecutive weighings vary less than 10 mg. Heat the sample in an oven at 100° for one hour, place in a desiccator for one-half hour and weigh. Repeat the application of heat for one-half hour, cooling and weighing, until a constant weight, varying not more than 0.5 mg. per gram of sample taken, is obtained. These values are shown in Table VII.

It will be noted that the values are less in the case of Tinctures of Belladonna, Hyoscyamus, and Nux Vomica, using the isopropyl alcohol in the menstruum, and noticeably higher in the case of Tincture of Stramonium, Camphorated Tincture of Opium, Compound Tincture of Cardamom and Aromatic Fluidextract of Cascara Sagrada, using the same alcohol.

Assays

All of the tinctures prepared were assayed by the official procedures. In addition, Tincture of Nux Vomica was assayed by the procedure proposed by Burlage and La Rocca (33) whereby the strychnine is separated from the brucine by means of an adsorption column. These values are shown in Table VIII.

The tinctures of Nux Vomica which are not defatted give lower results than the defatted ones by the U. S. P. XII method. This is probably due to the formation of emulsions in the extractions. These emulsions were more frequent, more stable and troublesome in working with samples in which isopropyl alcohol was used, and were obtained even with the defatted tinctures. The undefatted tinctures assayed by the chromatographic procedure, yielded higher values than the defatted tinctures because all of the fatty material was not removed by the adsorption column, thus permitting a probable reaction with the standard alkali.

Isopropyl alcohol yields a tincture of stramonium containing a surprisingly large amount of extractive, but low in total alkaloidal content. It is possible that this low value is due to an interference of some constituent(s) contained in the large amount of extractive.

TABLE I.—SPECIFIC GRAVITIES OF THE PREPARATIONS AND THEIR SOLVENTS

Solvent Concentration	Specific Gravity of Solvent at 25° Ethyl Alcohol	Specific Gravity of Solvent at 25° Isopropyl Alcohol	Preparation	Specific Gravity at 25° Ethyl Alcohol	Specific Gravity at 25° Isopropyl Alcohol	Increase or Decrease Ethyl Alcohol	Increase or Decrease Isopropyl Alcohol
Alcohol: Water, 3:1	0.882	0.867	Tincture of Belladonna	0.891	0.874	0.009	0.007
Alcohol: Water, 3:1	0.882	0.867	Tincture of Hyoscyamus	0.889	0.863	0.007	−0.004
Alcohol: Water, 3:1	0.882	0.867	Tincture of Stramonium	0.892	0.866	0.010	−0.001
Alcohol: Water, 3:1	0.882	0.867	Tincture of Nux Vomica[a]	0.890	0.870	0.008	0.003
Diluted Alcohol: Glycerin, 100:1	0.938	0.930	Camphorated Tincture of Opium	0.965	0.938	0.027	0.008
Diluted Alcohol: Glycerin, 19:1	0.939	0.944	Compound Tincture of Cardamom	0.958	0.939	0.019	−0.005
Alcohol: Water, 1:4	0.977	0.975	Aromatic Fluidextract of Cascara Sagrada	1.112	1.119	0.135	0.144
Alcohol: Water, 1:4	0.977	0.975	Fluidextract of Cascara Sagrada	1.073	1.081	0.096	0.106
...	...	...	Elixir of Phenobarbital	1.160	1.160	...	...
...	...	...	Compound Elixir of Pepsin	1.055	1.056	...	...
...	...	...	Elixir of Terpin Hydrate	1.069	1.077	...	...
...	...	...	Elixir of Terpin Hydrate with Codeine	1.067	1.070	...	...
...	...	...	Elixir of Three Bromides	1.249	1.258	...	...
...	...	...	Aromatic Elixir	1.087	1.083	...	...
...	...	...	Elixir of Five Bromides	1.284	1.301	...	...
...	...	...	Aromatic Spirit of Ammonia	0.898	0.875	...	...
...	...	...	Elixir of Iron, Quinine and Strychnine	1.084	1.097	...	...

[a] The specific gravity of the ethyl alcohol, hydrochloric acid, and distilled water used as a solvent in Tincture of Nux Vomica was 0.883; the isopropyl alcohol, acid, and distilled water mixture had a specific gravity of 0.877.

TABLE II.—*p*H'S OF THE PREPARATIONS AND THEIR SOLVENTS

Preparations	*p*H, Ethyl Alcohol	*p*H, Isopropyl Alcohol	*p*H of Solvent Ethyl Alcohol	*p*H of Solvent Isopropyl Alcohol
Tincture of Belladonna	5.55	5.28	6.05	5.85
Tincture of Hyoscyamus	5.55	5.36	6.05	5.85
Tincture of Stramonium	5.65	5.40	6.05	5.85
Tincture of Nux Vomica[a]	1.78	1.74	0.95	0.98
Camphorated Tincture of Opium	3.95	4.05	6.15	6.05
Compound Tincture of Cardamom	5.30	5.10	5.95	5.80
Aromatic Fluidextract of Cascara	7.80	7.05	6.25	6.05
Fluidextract of Cascara Sagrada	4.60	4.78	6.25	6.05
Elixir of Phenobarbital	4.60	4.70	..	..
Compound Elixir of Pepsin	4.30	4.20	..	..
Elixir of Terpin Hydrate	5.25	4.85	..	..
Elixir of Terpin Hydrate with Codeine	6.80	6.85	..	..
Elixir of Three Bromides	3.36	3.41	..	..
Aromatic Elixir	4.36	4.35	..	..
Elixir of Five Bromides	3.75	3.70	..	..
Aromatic Spirit of Ammonia	9.40	9.40	..	..
Elixir of Iron, Quinine, and Strychnine	3.45	3.34	..	..

[a] The *p*H given above as the solvent for Tincture of Nux Vomica was the acid-alcohol mixture used.

TABLE III.—PHYSICAL PROPERTIES OF THE PREPARATIONS—COLOR

Preparations	Color, Ethyl Alcohol	Color, Isopropyl Alcohol
Tincture of Belladonna	Dark greenish black	Dark greenish black
Tincture of Hyoscyamus	Dark greenish black	Dark greenish black
Tincture of Stramonium	Dark greenish black	Dark greenish black
Tincture of Nux Vomica	Clear deep amber	Clear deep amber
Camphorated Tincture of Opium	Clear brown	Clear brown
Compound Tincture of Cardamom	Dark red	Dark red
Aromatic Fluidextract of Cascara Sagrada	Dark brown	Dark brown
Fluidextract of Cascara Sagrada	Dark brown	Dark brown
Elixir of Phenobarbital	Bright red, cloudy	Clear bright red
Compound Elixir of Pepsin	Clear bright red	Clear bright red
Elixir of Terpin Hydrate	Pale straw	Straw
Elixir of Terpin Hydrate with Codeine	Pale straw	Straw
Elixir of Three Bromides	Clear bright red	Clear bright red
Aromatic Elixir	Colorless	Colorless
Elixir of Five Bromides	Clear dark brown	Clear dark brown
Aromatic Spirit of Ammonia	Clear yellow	Clear yellow
Elixir of I., Q., and S.	Clear bright green	Clear bright green

TABLE IV.—PHYSICAL PROPERTIES OF THE PREPARATIONS—CHANGES ON STANDING

Preparations	Changes, Ethyl Alcohol	Changes, Isopropyl Alcohol
Tincture of Belladonna	No apparent change	Residue on side of container
Tincture of Hyoscyamus	No apparent change	Residue on side of container
Tincture of Stramonium	No apparent change	Residue on side of container
Tincture of Nux Vomica	No apparent change	No apparent change
Camphorated Tincture of Opium	No apparent change	No apparent change
Compound Tincture of Cardamom	Precipitate on top of liquid	No change
Aromatic Fluid-extract of Cascara Sagrada	No change	No change
Fluidextract of Cascara Sagrada	No change	No change
Elixir of Phenobarbital	No change	Slight precipitate
Compound Elixir of Pepsin	No change	Light, flocculant precipitate
Elixir of Terpin Hydrate	No change	Changed from pale straw to amber color
Elixir of Terpin Hydrate with Codeine	No change	Changed from pale straw to amber color
Elixir of Three Bromides	No change	No change
Aromatic Elixir	Slightly cloudy	No change
Elixir of Five Bromides	Precipitate	Precipitate
Aromatic spirit of Ammonia	No change	No change
Elixir of Iron, Quinine, and Strychnine	No change	No change

TABLE V.—PHYSICAL PROPERTIES OF THE PREPARATIONS—ODOR

Preparations	Odor, Ethyl Alcohol	Odor, Isopropyl Alcohol
Tincture of Belladonna	Tobacco-like	Tobacco-like
Tincture of Hyoscyamus	Tobacco-like	Tobacco-like
Tincture of Stramonium	Strongly tobacco-like	Strongly tobacco-like
Tincture of Nux Vomica	Slightly sweet	Faint odor of the alcohol
Camphorated Tincture of Opium	Characteristic	Characteristic
Compound Tincture of Cardamom	Cinnamon-like	Cinnamon and of the alcohol
Aromatic Fluid-extract of Cascara Sagrada	Heavy sweet odor	Heavy, less sweet odor
Fluidextract of Cascara Sagrada	Bitter, pungent	Bitter, and of the alcohol
Elixir of Phenobarbital	Orange-like	Faint odor of the alcohol
Compound Elixir of Pepsin	Sweet, orange-like	Sweet, faintly of alcohol
Elixir of Terpin Hydrate	Sweet	Faintly of the alcohol
Elixir of Terpin Hydrate with Codeine	Sweet	Faint odor of the alcohol
Elixir of Three Bromides	Odor of benzaldehyde	Odor of benzaldehyde
Aromatic Elixir	Odor of orange	Faint odor of the alcohol
Elixir of Five Bromides	Sickening sweet odor	Less sickening sweet odor
Aromatic Spirit of Ammonia	Characteristic	Characteristic
Elixir of Iron, Quinine, and Strychnine	Orange-like	Less orange-like

TABLE VI.—PHYSICAL PROPERTIES OF THE PREPARATIONS—TASTE

Preparations	Taste, Ethyl Alcohol	Taste, Isopropyl Alcohol
Tincture of Belladonna	Alcoholic	Slightly burning
Tincture of Hyoscyamus	Alcoholic	Slightly burning
Tincture of Stramonium	Strongly tobacco-like	Tobacco-like, burning
Tincture of Nux Vomica	Very bitter	Burning, bitter
Camphorated Tincture of Opium	Aromatic, burning	Aromatic, less burning
Compound Tincture of Cardamom	Aromatic	Slightly burning
Aromatic Fluid-extract of Cascara Sagrada	Syrupy, sweet	Syrupy, sweet
Fluidextract of Cascara Sagrada	Very bitter	Very bitter, burning
Elixir of Phenobarbital	Sweet, aromatic	Aromatic, faintly burning
Compound Elixir of Pepsin	Faintly alcoholic	Faintly alcoholic
Elixir of Terpin Hydrate	Sweet, aromatic	Aromatic, faintly burning
Elixir of Terpin Hydrate with Codeine	Slightly bitter	Slightly bitter and burning
Elixir of Three Bromides	Very saline	Less saline
Aromatic Elixir	Aromatic	Slightly burning
Elixir of Five Bromides	Very saline	Less saline
Aromatic Spirit of Ammonia	Burning	Burning
Elixir of Iron, Quinine, and Strychnine	Very bitter	Bitter

TABLE VII.—TOTAL EXTRACTIVE IN THE PREPARATIONS

Preparations	Total Extractive, Gm./100 Cc. Ethyl Alcohol	Total Extractive, Gm./100 Cc. Isopropyl Alcohol	Description of Residue Ethyl Alcohol	Description of Residue Isopropyl Alcohol
Tincture of Belladonna	2.4974	2.4112	Dark green	Black
Tincture of Hyoscyamus	1.8206	1.4379	Light brown	Dark brown-green
Tincture of Stramonium	0.3727	2.5359	Green-brown	Dark brown
Tincture of Nux Vomica	1.7423	1.6859	Brown	Brown
Camphorated Tincture of Opium	8.1509	8.4052	Brown	Brown
Compound Tincture of Cardamom	5.8649	6.7965	Deep red oily liquid	Deep red oily liquid
Aromatic Fluidextract of Cascara Sagrada	34.6987	38.4526	Dark brown	Dark brown

TABLE VIII.—ANALYTICAL VALUES FOR THE TINCTURES

Preparations	Total Extractive Ethyl Alcohol, Gm./100 Cc.	Alkaloids, Ethyl Alcohol, Gm./100 Cc.	Total Extractive Isopropyl Alcohol, Gm./100 Cc.	Alkaloids, Isopropyl Alcohol, Gm./100 Cc.
Tincture of Nux Vomica, U. S. P., undefatted	1.7423	Strychnine 0.11587 0.11566	1.6859	Strychnine 0.09771 0.10293
Tincture of Nux Vomica, U. S. P. XII, defatted	...	Strychnine 0.14736 0.14715	...	Strychnine 0.13399 0.13364
Nux Vomica, chromatographic assay, undefatted	...	Strychnine 0.18480 0.18450	...	Strychnine 0.22338 0.22251
Nux Vomica, chromatographic assay, defatted	...	Strychnine 0.15717 0.15717	...	Strychnine 0.22048 0.22137
Tincture of Belladonna, U. S. P. XII procedure	2.4974	0.03907 0.03964	2.4112	0.03907 0.39940
Tincture of Stramonium, U. S. P. XII assay	0.3727	0.03791 0.03704	2.5359	0.02858 0.02848
Tincture of Hyoscyamus, U. S. P. XII procedure	1.8206	0.00366 0.00360	1.4379	0.00469 0.00466
Camphorated Tincture of Opium, U. S. P. XII procedure	8.1509	0.04994 0.04983 0.04975	8.4052	0.04975 0.04983 0.04966

SUMMARY AND CONCLUSIONS

1. From the standpoint of ease of preparation, isopropyl alcohol is of advantage as a solvent in eight of the preparations studied.

2. The odor of isopropyl alcohol is an objectionable one and is not easily masked; however, benzaldehyde seems to accomplish this quite effectively.

3. Isopropyl alcohol compares favorably with ethanol in preparing Camphorated Tincture of Opium by maceration, and Tincture of Belladonna by percolation. In Tincture of Hyoscyamus, it proved to be a better solvent of the total alkaloids.

4. In the case of the tincture of Nux Vomica, the tincture containing isopropyl alcohol showed less strychnine when assayed by the Pharmacopœial method than that containing ethyl alcohol, but showed a higher strychnine content using the chromatographic method of assay.

5. The alcohol is apparently an unsatisfactory solvent for the preparation of Tincture of Stramonium because of the low yield of alkaloids and the high yield of extractive.

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Book Review

Goethe's Botany, by AGNES ARBER. The Chronica Botanica Company, Waltham, Mass., and G. E. Stechert and Co., New York, 1946. 63 pages. 17.5 × 26 cm. 6 illus. Price $2.00.

It is significant of the frantic longing of present day mankind for tracing back all the confusing complexity of life to some fundamental bases on which the edifice of an all-embracing coordinated something—a "ONE WORLD"—could be erected, that similar efforts are given renewed attention.

It was in 1790 that the German poet Goethe's *Attempt To Interpret the Metamorphosis of Plants* appeared. Its purpose was to create (and to prove) the concept of a fundamental ["*Ur*"] plant from which the whole body of plants now in existence may be derived. The discussion of this astounding publication has never ceased. It gained, however, a new impulse after the first world war. There have appeared during the last twenty-five years a number of German treatises on this subject and a remarkable English one (by Ch. Sherrington in 1942) and, while there was only one English version (published in 1863) of Goethe's pamphlet until quite recently another one was published in 1937 and, but nine years later, the one under consideration.

The translation by Agnes Arber comes close not only to the letter, but also to the spirit, of Goethe's pamphlet. Her introduction, finally, merits the highest praise possible, namely the statement that it really means what its title says, i.e., that the reader is actually "introduced" into the problem and the attempts at its explanation. The following quotation reveals Miss Arber's idea of Goethe's type concept and its place in Goethe's general philosophy:

"In Goethe's eyes the type principle was the clue to the interpretation of animals as well as plants . . . None of Goethe's thinking was ever isolated from his whole mental activity, and the type concept, or, more widely the idea of *Ur* [fundamental] phenomena, was to him a clue to be followed not in science merely; it was, rather, one of the keys which gave him the freedom of the universe as a whole."

Miss Arber quotes a book by Julius Schuster published in 1924 and entitled *Goethe, die Metamorphose der Pflanzen, mit dem Original bildwerk* (Goethe, the metamorphosis of plants, with the original illustrations). She does not mention the same author's essays on "*Idealistische Morphologie-als Gegenwartsproblem*" (Idealistic Morphology as a Problem of Our Time), published in 1928, and "*Goethe und die Biologie*" (1932). It is the last publication that Schuster gives the following extremely German explanation, which is most significant:

"Goethe's *Ur* [fundamental] phenomenon itself is not a reality anymore, but a visionary abstraction, an abstract picture. This abstraction, however—this generalization—sought by Goethe is by no means identical with the common idea of something general: It still remains something that is individual. And things individual cannot be explained by means of concepts but only by perception—by pictures. Goethe requires that the individual, while brought into line with the continuous sequence of happenings, retain its identity within this order. It must *be* and *remain* individual, if the general shall truly be represented to him. It is the problem of individualizing perception which we are facing here."

In conclusion it may be stated that in the astounding figure of Goethe mankind has been given one of the very rare representatives of the race, human enough to be moved by all the problems of man and great enough to carry them to the highest level of consideration. Thus, on the centenary (1932) of his death there was scarcely one branch of science, art, and thought that did not testify to the influence of Goethe. For pharmacy this was done by this reviewer in an article "*Goethe und die Pharmazie*" [*Pharm. Ztg.*, 77, 333–39(1932)].—GEORGE URDANG.

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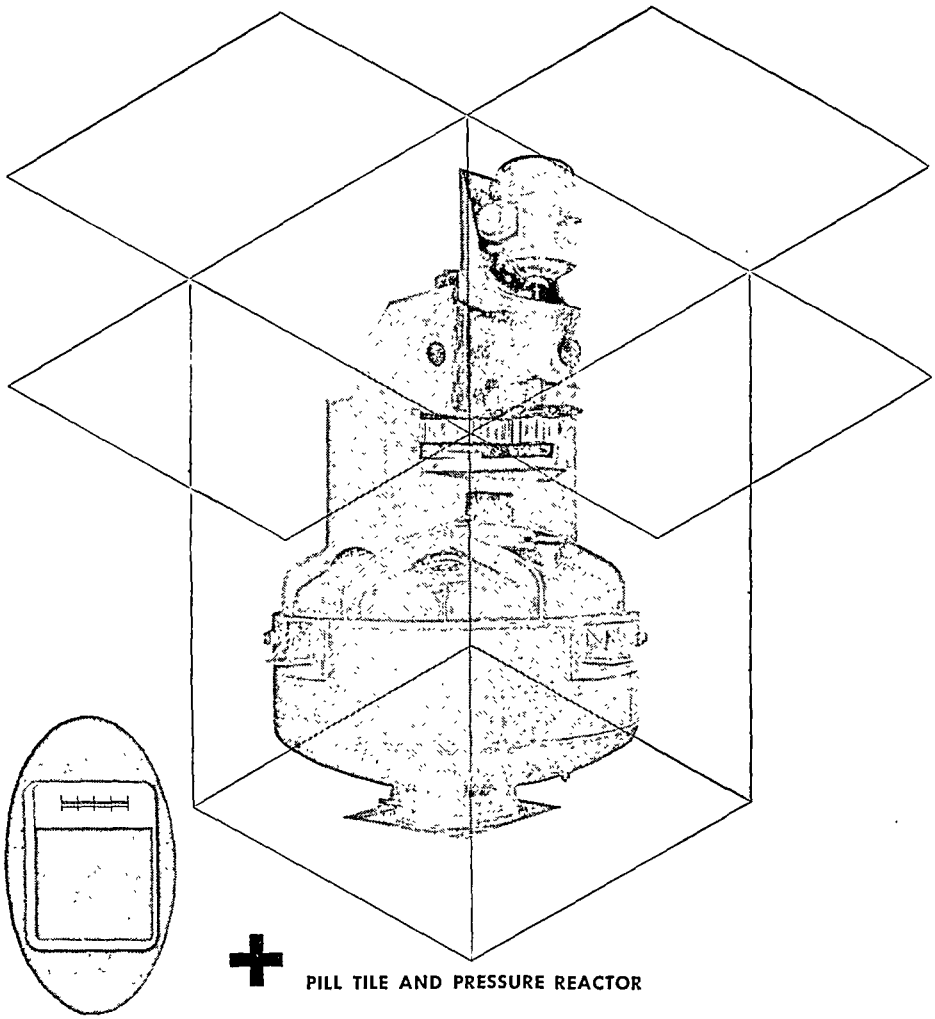

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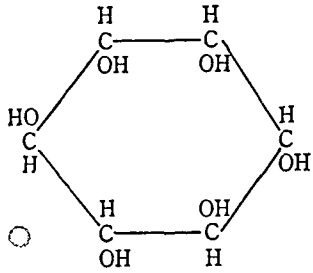

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Barbiturates—Are They Narcotics?*

By SAMUEL W. GOLDSTEIN†

The distinction between narcotics and hypnotics is discussed. Differences in authoritative opinions concerning the inherent dangers of the barbiturates are indicated. Data based on hospital records to barbiturate habituation are presented and the need for legislation regulating barbiturate distribution is emphasized. The author concludes that legally barbiturates should not be classified as narcotics.

THE PROBLEM of adequate control of the distribution of barbiturates is recognized by many. The seriousness of the situation is shown by statistical data (1–3). The solution of the problem is being diligently sought by professional and lay groups. The medical and pharmaceutical groups seek to achieve control of barbiturate distribution by sponsoring effective uniform state laws. The lay groups seek to have the barbiturates legally classified as narcotics and made subject to all the regulations applying under the Federal Narcotic Act. Responsible public health officials are acutely aware of the barbiturate problem and agree that legislation of some kind is necessary. Dr. Paul Dunbar, U. S. Commissioner of Foods and Drugs, during a recent discussion of control of barbiturate distribution, stated: "A federal law would be too complicated and drastic. Uniform state laws would be more satisfactory" (4).

NARCOTICS AND HYPNOTICS

The barbiturates are generally referred to as hypnotics. What is the difference between narcotics and hypnotics? Medical dictionaries (5) list the two terms without cross-references. The definitions for "narcotic" state that stupor or sleep is produced and that pain is relieved. "Hypnotic" is defined as a drug that induces sleep. "True hypnotics" produce normal sleep. Other hypnotics include chloroform, opium, and other drugs that have an anodyne effect. Apparently the lexicographers interpret the two terms as having separate meanings. European medical writers customarily use the term "narcotic" to include hypnotics and frequent references are made to the barbiturate narcotics. American medical literature contains many statements about the hypnotic effect and the narcotic effect of

* Received Jan. 14, 1947, from the State of Maryland Department of Health, Baltimore.
† Pharmaceutical Chemist, State of Maryland Department of Health.

barbiturates but almost always refers to the barbiturates as hypnotics. Finally we have the legal definition of "narcotic drugs" as "coca leaves, opium, cannabis, and every substance not chemically distinguishable from them" (6).

Undoubtedly the main reason for passing the Federal Narcotic Act in 1914 was the rapid increase in the number of addictions to opiates and coca derivatives. During the period that this law has been in force the term "narcotic" has been accepted as applying to opiates, coca derivatives, and cannabis or marihuana. The question now being raised is this: Should the barbiturates be added to this group of drugs?

Among physicians there are honest differences of opinions as to the dangerous nature of barbiturate medication. Shelton (7) states ". . . . . the barbituric acid derivatives produce severe pathological changes in the organism when given in large doses over any moderate length of time. Even the small therapeutic doses when frequently repeated cause a moderate amount of tissue destruction. The barbiturates have an affinity for nervous tissue cells and destruction is most marked in the higher centers of the central nervous system." G. W. Robinson, Sr. (8) states, "In the treatment of mental ill health, barbiturates have a place, but only to meet emergencies and temporarily to relieve the patient of insomnia and mental distress. It is a form of medication which may be temporarily helpful in relieving symptoms but if continued over a period of days or weeks will do much more harm than good." Grogan (9) discusses the various uses of barbiturates in mental hospitals. He describes the results obtained, and continues: "All this is attained with no harm to the patient. I have never seen barbiturates, used in the ways described, bring about addiction to the drug. The few cases of barbiturate addiction that have come to my attention had an extremely apparent underlying psychiatric basis for their addiction." Grogan stresses the point that in all cases where barbiturates are administered over a long period of time, the minimum effective dose for the patient should be established and maintained.

Moore and Gray (10) in their discussion of patients who were believed to have a psychosis due to drugs (including barbiturates) or exogenous chemical poisons, state: "Many patients cleared mentally in a short time after withdrawal of the drugs. A few grew steadily worse, particularly those who had been using morphine and opium, probably due to the onset of withdrawal symptoms which are characteristically seen in users of the narcotic phenanthrene derivatives. . . Present knowledge concerning dependence on drugs is negative, for this phenomenon appears only upon withdrawal of drugs. The phenanthrene derivatives (morphine, etc.) appear to produce more dependence than do some of the coal tar drugs (barbiturates, for example). Persons who use drugs are motivated by the same mechanisms as obtain in alcoholism, and it is pointed out that drugs, other than alcohol, do not approach the importance of that substance as contributory factors in the production of mental disease."

BARBITURATE ADDICTION

A large proportion of the barbiturate addiction cases are associated with opiate addiction. Many of these will return to complete dependence upon opiates as soon as these drugs become available. The sad fact is that the opiates will become available in spite of the truly valiant work of our too small group of federal narcotic control agents. If some other countries were nearly as diligent as the United States in the curbing of traffic in opiates, cocaine, and cannabis, the already admirable results of the efforts of our enforcement agents would be even more spectacular. Nevertheless, during the period of 1940–1945, which included the war years when importation of opiates was practically eliminated, the addictions recorded in certain hospitals showed that the ratio of barbiturate addictions to total addiction cases did not increase.

While the hospital records of addictions give a picture that is far from complete, they are at present the most reliable data available. Furthermore, the comparison of hospital data from two different periods gives an indication of the frequency of occurrence

of addictions due to all drugs and of barbiturate habituation.

Hambourger (2) reported that for the decade 1928–1937 thirteen hospitals listed eighty-five cases under barbiturate addiction, an incidence of about one case for every 15,000 admissions. He reported more complete data for the following five hospitals: Baylor University, Dallas, Texas; Boston City, and Peter Bent Brigham, Boston; Presbyterian, New York; Michael Reese, Chicago. These hospitals had combined admissions for all causes of 761,923 cases. They had 398 cases of addiction to all drugs (excluding chronic alcoholism), forty-three of which, or 10.8 per cent. involved barbiturates; about one of every nine addiction cases.

The present author has compiled data for the six-year period 1940–1945, from the records of the following hospitals: Baylor University, Dallas, Texas; Boston City, Boston; Cleveland City, Cleveland; Presbyterian, New York; Presbyterian, Michael Reese, and University Clinics, Chicago; Walter Reed General, Washington, D. C.; St. Mary's, St. Louis; Baltimore City, Johns Hopkins, Mercy, Sinai, Union Memorial, and University, Baltimore. These hospitals had combined admissions for all causes of 1,138,887 cases. They had 715 cases of addiction to all drugs (excluding chronic alcoholism), sixty-eight of which, or 9.5 per cent, involved barbiturates; about one of every eleven addiction cases. The incidence of barbiturate addiction in the total admissions was one case in every 17,000 admissions. These data show that the frequency of barbiturate addiction compared to total admissions and to addictions to all drugs decreased in the 1940–1945 period. They also show that the frequency of addictions to all drugs compared to total admissions increased from one in every 1900 admissions during 1928–1937 to one in every 1600 admissions during 1940–1945.

This does not mean that the entire barbiturate situation improved during the years following 1937. Data published by Goldstein (1) show that this is far from true. But the present data emphasize the far lesser danger of addiction by barbiturates than by other habit-forming drugs, especially those legally classified as narcotics.

WITHDRAWAL SYMPTOMS

While continued daily use of barbiturates can lead to habituation, the almost complete absence of withdrawal reactions is in marked contrast to the reactions produced when drugs are withheld from opiate addicts. Barbiturates do not produce the euphoria or sense of well-being in the same sense or extent that is produced with opiates, cocaine, or cannabis. Barbiturates are not analgetic or pain-relieving, as are the opiates. The habituation developed on long usage of barbiturates is broken very easily compared with the addictions to opiates and cocaine. The barbiturates, when used alone, do not cause the psychic reactions which lead marihuana users to commit criminal acts. The continued use of opiates leads to the development of such a tolerance to the drug that addicts who desire euphoria are driven to the use of ever-increasing doses, sometimes ten or twenty times more than would be fatal to an unaccustomed individual (11). With barbiturates, a certain amount of physical adaptation is readily acquired, as with alcohol; but there is little real tolerance for large doses. Prolonged use of barbiturates by psychologically susceptible persons may cause the development of psychical degeneration and mania, as with alcohol, but these are often absent after long continued daily administration. With opiates, daily use will lead to addiction in ten days with susceptible individuals, and in twenty to twenty-five days in those with normal emotional stability (12).

The following case history, submitted by I. Kaplan, M.D. (13), is an example of the rational use of barbiturates over an extended period of time. A 60-year old male suffered an attack of coronary thrombosis. To insure complete rest, he was given 1 grain of phenobarbital sodium every four hours and $1^1/_2$ grains of pentobarbital sodium each night for a period of four weeks. Following this, the daily administration was limited to the nightly dose. The case was complicated by the development of an embolism during

the third week. After nine weeks of daily barbiturate administration, the improved patient showed no signs of drug intoxication, but showed sufficient drug dependence to demand his "sleeping pill" each night. During the tenth and eleventh weeks the patient, on his physician's advice, cut his sleeping pills down to one every other night. When he became ambulatory, during the twelfth week, the administration of barbiturates was discontinued with no apparent ill effect to the patient.

In 1940, G. W. Robinson, Jr., M.D. (14) discussed the seriousness of the barbiturate situation, and made the following statements: "It should not be necessary to wait for lawmakers [to correct the condition]. The physicians of the country are largely responsible for the situation. We have been too gullible in accepting the statements of the manufacturers and, worse if possible, we have passed those statements on to the laymen. Patients have been told by the family doctors, the consulting specialists, their nurses, and others in authority that they can take this medicine as long as they want to, that it will not hurt them and will not form a habit. There may have been a time when the knowledge of the dangers of these drugs was so limited that such statements could be made with sincerity but that day is past, ..."

Robinson may have been too harsh with his colleagues, but surely many physicians, pharmacists, and nurses still must be convinced of the dangers in barbiturate usage. The educational program should be pushed. The legislative program is being activated. The question as to how severe the legislative restrictions must be to achieve proper control has been studied and reported by Fischelis (15). The Legislative Committee of the AMERICAN PHARMACEUTICAL ASSOCIATION and the National Drug Trade Conference have endorsed a model Uniform State Barbiturate Act. The various state pharmaceutical and medical societies are studying this bill in order to make any necessary changes before its introduction to the state legislatures for enactment. This legislation should go far to correct the faults in the present system of barbiturate distribution.

The classification of barbiturates as narcotics may have some valid basis when considered only from a pharmacological viewpoint. But, taking the foregoing data into consideration, the answer to the question: "Should the barbiturates be classed as narcotics legally?" is "No."

SUMMARY

1. The ambiguous uses of the terms "narcotic" and "hypnotic" are discussed.

2. The differences in opinion among physicians regarding the dangerous nature of the barbiturates is indicated.

3. Hospital records for the periods 1928–1937 (I) and 1940–1945 (II) yielded the following data:

(*a*) Addictions to all drugs (chronic alcoholism excepted): Period I. One in every 1900 admissions. Period II. One in every 1600 admissions.

(*b*) Addictions to barbiturates: Period I. One in every 15,000 admissions. Period II. One in every 17,000 admissions.

(*c*) Relationship of barbiturate habituation to total addictions: Period I. One in every nine, or 10.8 per cent. Period II. One in every eleven, or 9.5 per cent.

4. The need for effective legislation to regulate barbiturate distribution is emphasized.

5. The question of legally classifying barbiturates as narcotics is answered in the negative.

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Hydrophilic Properties of Certain Ointment Base Constituents*

By ALFRED HALPERN† and LOUIS C. ZOPF‡

A series of saturated C_{10} to C_{18} fatty alcohols were studied for their effect on the hydrophilic properties of petrolatum. An optimum concentration at which each fatty alcohol exerts its maximum effect is noted. Cetyl alcohol has the lowest optimum value, but octadecyl alcohol (stearyl alcohol), having a slightly higher optimum value, exerts the strongest effect on the hydrophilic properties of petrolatum. The values obtained by two methods are compared and the practical value of this property of the fatty alcohols is discussed. A comparative study of these alcohols in bases analogous to that described in the Swiss Pharmacopoeia V is made.

A METHOD for the extemporaneous formulation of a hydrophilic ointment base is of decided advantage to the pharmacist and physician. The need frequently arises for a base that will incorporate a definite quantity of water or a solution of a medicament. While many excellent bases that will take up varying amounts of water have been reported, these bases, for the most part, are fixed in their water content. There is very little flexibility in their hydrophilic properties. We realized the possibilities toward improved ointment therapy through the development of a system of formulation, whereby the amount of water taken up by an ointment base can be predicted and how such a base might be altered to accommodate varying amounts of water without qualitatively changing the product. A knowledge of the effects of varying concentrations of ointment base constituents on the hydrophilic properties of the base as a whole would result in such a system. Thus, by quantitatively altering these agents, a base could be constructed to accommodate a predetermined amount of water or solution of a medicament.

This study was undertaken to extend the work of Casparis and Meyer (1) and to investigate the hydrophilic properties of a homologous series of saturated fatty alcohols. We investigated the saturated fatty alcohols from C_{10} to C_{18}. The "water numbers" of the individual alcohols were noted (Table IV) and a study of the combined effects of the fatty alcohol and wool fat on the water-absorbing properties of petrolatum was also carried out.

We wish to emphasize that the resulting preparations of the fatty alcohols and petrolatums are not to be construed as a recommended ointment base, nor do we propose that the hydrated mixture be used in such a manner, without further adjustment. It is noted, however, that the Swiss Pharmacopœia V has official a base that is analogous to the fatty alcohol-wool fat-petrolatum group (Part II) (5).

PART I

In 1935 Casparis and Meyer published their results of a study of the hydrophilic properties of certain ointments and ointment bases (1). They described a method for determining the "water number" of a fat or ointment base. Of particular interest is their statement that the water number of a mixture is roughly the sum of the increases produced by the individual components. Among the substances investigated, cetyl alcohol was found to increase the water number of petrolatum three to four times. An optimum concentration of the cetyl alcohol in petrolatum was noted by the investigators. At this optimum, cetyl alcohol causes its maximum potentiation of the water-absorbing properties of petrolatum.

The "water number" as defined by Casparis and Meyer, is the largest amount of water which 100 Gm. of an ointment base or fat will hold at normal temperature (20°). Their method of determining the water number is as follows:

Method 1—"The incorporation of the maximum amount of water was effected in a mortar by adding water to the melted base and triturating until cool. If no water remained, more was added in small amounts until no more was taken up. The ointment was transferred to a jar and kept in a refrigerator for several hours at about 0°. It was then al-

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lowed to come up to room temperature and rubbed on a slab with a spatula. This procedure was repeated until no more water exuded. The water present in a 5- or 10-Gm. sample of the base was determined by the method of Pritzker and referred to 100 Gm. of base."(2).

The water number is calculated in the following manner:

Weighed amount of saturated base $= x$
Water content $= y$
Weight of base alone $= (x - y)$
Water number $= \dfrac{100y}{(y - x)}$

The water numbers of various concentrations of a homologous series of fatty alcohols (Table I) in petrolatum were determined by this method (Table II). The study was limited to concentrations up to 15% of the fatty alcohol in petrolatum because our objective was to learn the effects of the fatty alcohols at minimal concentrations. We were not concerned with any further physical property that is contributed by the fatty alcohols to the base at higher concentrations.

TABLE I.—FATTY ALCOHOLS TESTED

Fatty Alcohol	Formula	Grade[a]
Decanol	$C_{10}H_{21}OH$	Technical
Lauryl alcohol	$C_{12}H_{25}OH$	Technical
Myristyl alcohol	$C_{14}H_{29}OH$	Unknown
Cetyl alcohol	$C_{16}H_{33}OH$	Cosmetic
Stearyl alcohol (Stenol)	$C_{18}H_{37}OH$	Cosmetic
Petrolatum (yellow)		U. S. P.
Petrolatum (white)		U. S. P.
Wool fat		U. S. P.

[a] In no case were the physical properties observed of the fatty alcohols those given in the Handbook of Chemistry and Physics.

another method was employed to determine the water numbers of the various petrolatum fatty alcohol mixtures.

Method 2.—A 10-Gm. portion of the appropriate petrolatum-fatty-alcohol mixture was triturated in a mortar with water delivered from a burette 0.5 cc. at a time. When no more water was taken up, the mixture was chilled for an hour in an ice bath. It was then allowed to warm to room temperature and was levigated on a slab. If water was exuded by levigation, the process was repeated on a fresh sample, using 0.1 cc. less of water. This was repeated until a level was reached at which no water could be exuded by levigation. As a further check, the amount of water taken up by 10 Gm. of the mixture was referred to 100 Gm. and this value checked by adding this quantity of water to 100 Gm. of the mixture and treating as above. The values recorded are those for 100 Gm. of the mixture (Table III).

DISCUSSION

The values obtained indicate that there is a definite relationship between the concentration of the fatty alcohol and the amount of water held by the mixture. It is important to note that the water number is specific for the fat. Thus Casparis and Meyer report (1–3) that while 4% of cetyl alcohol in petrolatum raised the water number of petrolatum from (9.3–15.6) to (38.8–51.5), a 3% of cetyl alcohol in hydrogenated peanut oil raised the water number of the peanut oil from 75.4 to 185.7. Lard had its water number raised from 7.5 to 244.9 by a 3% concentration of cetyl alcohol. For this reason, the fat phase should always be indicated in any discussion of the water number of a substance.

TABLE II.—WATER NUMBERS OF FATTY ALCOHOL-PETROLATUM MIXTURES DETERMINED BY METHOD 1

	Concentration in White Petrolatum											
Fatty alcohol	1%	2%	3%	4%	5%	6%	7%	8%	9%	10%	12.5%	15%
Decanol	10.14	11.2	12.75	13.26	14.61	15.43	16.15	16.8	17.2	17.8	18.1	16.2
Lauryl alcohol	14.1	17.2	18.3	19.4	21.0	22.7	23.5	24.6	25.4	23.2	21.4	19.2
Myristyl alcohol	21.7	22.8	26.5	28.7	31.2	30.8	32.5	34.8	30.3	31.4	28.0	24.1
Cetyl alcohol	31.1	36.2	38.0	38.5	36.1	36.4	35.1	34.0	33.2	31.2	30.6	29.5
Stearyl alcohol	32.0	35.1	39.3	41.2	42.5	43.2	40.1	40.5	39.7	38.0	35.6	33.2
	Concentration in Yellow Petrolatum											
Fatty alcohols	1%	2%	3%	4%	5%	6%	7%	8%	9%	10%	12.5%	15%
Decanol	10.7	12.1	12.9	13.5	14.1	15.6	16.7	17.1	17.9	18.8	19.8	16.9
Lauryl alcohol	14.2	16.1	17.3	19.5	20.6	22.3	24.0	25.8	27.1	25.2	22.1	19.3
Myristyl alcohol	22.6	24.5	27.1	28.5	30.8	31.3	33.5	34.6	32.1	30.2	27.4	25.1
Cetyl alcohol	32.6	35.1	38.2	40.2	38.8	37.1	36.0	34.1	32.6	31.1	29.8	29.1
Stearyl alcohol	35.1	38.2	40.1	42.6	44.3	45.2	41.2	40.3	39.0	38.2	36.0	34.0
Water number of White petrolatum				9.0								
Water number of Yellow petrolatum				10.2								

The optimum concentration of the fatty alcohol in the petrolatum derived by this method was noted in Table IV.

As the authors (Casparis and Meyer) make the statement that this method does not have the exactness of other analytical methods since various interfering factors cannot be well controlled (1), The purity of the fatty alcohol and the petrolatum is important in the application of the water number to extemporaneous formulation. A range of values has been obtained depending on the quality of the fatty alcohol and the petrolatum used. In order to standardize our results and eliminate as much as possible this source of variation, we used

TABLE III.—WATER NUMBERS OF FATTY ALCOHOL AND PETROLATUM MIXTURES DETERMINED BY METHOD 2

Fatty alcohol	Concentration in White Petrolatum 1%	2%	3%	4%	5%	6%	7%	8%	9%	10%	12.5%	15%
Decanol, $C_{10}H_{21}OH$	11.3	12.1	13.5	14.2	15.1	15.1	15.7	16.1	17.0	17.2	17.8	15.1
Lauryl alcohol, $C_{12}H_{25}OH$	15.2	18.3	18.5	19.7	21.8	22.5	23.0	24.1	25.2	22.7	20.3	19.0
Myristyl alcohol, $C_{14}H_{29}OH$	21.0	23.2	26.1	29.0	30.1	30.3	31.3	32.8	30.0	28.1	26.0	23.2
Cetyl alcohol, $C_{16}H_{33}OH$	30.0	35.5	37.8	38.2	38.0	37.6	37.0	36.1	36.3	35.2	32.1	32.4
Stearyl alcohol, $C_{18}H_{37}OH$	33.1	36.5	38.2	39.0	40.7	42.3	42.0	41.3	40.2	38.7	37.5	36.8
Fatty alcohol	**Concentration in Yellow Petrolatum 1%**	**2%**	**3%**	**4%**	**5%**	**6%**	**7%**	**8%**	**9%**	**10%**	**12.5%**	**15%**
Decanol, $C_{10}H_{21}OH$	11.2	12.7	13.8	14.8	16.0	16.3	17.1	17.8	18.0	18.4	19.1	17.8
Lauryl alcohol, $C_{12}H_{25}OH$	15.5	17.8	18.7	19.5	21.6	22.8	23.5	24.8	26.0	25.1	24.2	23.0
Myristyl alcohol, $C_{14}H_{29}OH$	20.0	23.4	25.6	28.2	30.3	30.8	32.0	33.2	31.2	29.2	27.1	24.8
Cetyl alcohol, $C_{16}H_{33}OH$	31.5	36.1	41.2	43.5	40.1	39.8	38.1	36.5	36.0	35.0	34.1	33.8
Stearyl alcohol, $C_{18}H_{37}OH$	35.0	38.2	39.3	41.2	43.1	45.3	43.0	41.6	42.0	40.0	38.2	37.1
Water number of white petrolatum			9.5									
Water number of yellow petrolatum			10.8									

a petrolatum described as meeting U. S. P. XII specifications and fatty alcohol commonly sold as the dermatological grade. However, it is important to note that in some cases these were designated as "technical grade." The purities of the fatty alcohols used are described in Table I.

TABLE IV.—OPTIMUM CONCENTRATION AND WATER NUMBERS OF FATTY ALCOHOLS

Fatty Alcohol	Method 1 Optimum Concentration, %	Method 1 Water Number	Method 2 Optimum Concentration, %	Method 2 Water Number
White Petrolatum				
Decanol	12.5	18.1	12.5	17.8
Lauryl alcohol	9.0	25.4	9.0	24.1
Myristyl alcohol	8.0	34.8	8.0	32.8
Cetyl alcohol	4.0	38.5	4.0	38.2
Stearyl alcohol	6.0	43.2	6.0	42.3
Yellow Petrolatum				
Decanol	12.5	19.8	12.5	19.1
Lauryl alcohol	9.0	27.1	9.0	26.0
Myristyl alcohol	8.0	34.6	8.0	33.2
Cetyl alcohol	4.0	40.2	4.0	43.5
Stearyl alcohol	6.0	45.2	6.0	45.3

Of the series studied, cetyl alcohol had the lowest optimum concentration and stearyl alcohol caused the greatest potentiation of the water number of petrolatum. The water number of petrolatum apparently increased with the increase in the length of the carbon chain. The concentration necessary to cause the maximum increase has its minima at a carbon chain length of 16. Thus, the fatty alcohols tested on both sides of cetyl alcohol require a greater concentration to exert their maximum effect on the water number of petrolatum. We hesitate to draw conclusions from a minimal amount of data; therefore we merely report these observations as properties of the fatty alcohols tested and not as a generalization on the fatty alcohols of the type $C_nH_{(2n+1)}OH$. We are continuing the investigation of the relationship of the lipophilic group to the hydrophilic group with regard to the effect on the water number of ointment base constituents.

Of the alcohols studied, decanol and lauryl alcohol possess a characteristic odor which detracts from their use in ointments. Myristyl, cetyl, and stearyl alcohols do not have this objectionable feature. All emulsions resulting from this study were stable "water-in-oil" types. "Sweating" was evidenced in the saturated mixtures kept at 45° overnight. However, preparations containing 3% to 5% less water than that indicated by the water number showed no "sweating" at this temperature. No "sweating" was observed with the saturated mixtures kept at room temperature (25°). This would seem to indicate that the water number is influenced by temperature.

PART II

One of the purposes of this study was to develop a means of extemporaneous formulation of hydrophylic ointment bases. Of particular interest in this regard is the statement that "the water number of a mixture is roughly the sum of the water numbers of its components" (2).

We decided to study the effects of a mixture of two fatty alcohols and petrolatum and wool fat-fatty alcohol-petrolatum on the water number of petrolatum. The methods used to determine the water number of these mixtures were those described in Part I of this paper. The fatty alcohols were used at their optimum concentrations (A) and the wool fat-fatty alcohol-petrolatum series was patterned after that described in the Swiss Pharmacopœia V (B). The type of formulas used were:

(A) Fatty alcohol I.....Optimum concentration
Fatty alcohol II....Optimum concentration
Petrolatum *q. s.*....100 Gm.

(B) Wool fat.....10 Gm.
Fatty alcohol.....Optimum concentration
Petrolatum *q. s.*....100 Gm.

Casparis and Meyer investigated the Unguentum Cetylicum of the Swiss Pharmacopœia V and found

that it had a water number of 81.1 to 108.3. This base contains the optimum concentration of cetyl alcohol (4%) in wool fat (10%) and petrolatum (86%). The range of the water number was attributed to the type of petrolatum used.

Professor Rupp (4), studying a base containing myristyl alcohol in the place of cetyl alcohol in the above formula (B), found that it took up about 10% less water than the cetyl alcohol preparation. He further states that myristyl alcohol gives ointments which are inferior to cetyl alcohol as regards the taking up of water.

We repeated these determinations merely to complete our series. It was found that introducing the maximum amount of water (i.e., the amount indicated by the water number) produced a preparation that did not possess all of the physical properties desired in an ointment base. A preparation that was extremely soft resulted, particularly so in the mixtures containing over 110% of water, based on the quantity of fat phase. By using 10% to 15% less water than is indicated by the water number a preparation having an increased pharmaceutical elegance resulted.

The water number of a mixture of fatty alcohols and petrolatum (Formula A) is not the sum of the individual water numbers. There was some potentiation but this was not constant. In the cases where decanol and lauryl alcohol were used, a slight decrease in the water number was noted. The mixture of myristyl-cetyl, myristyl-stearyl, and cetyl-stearyl alcohols showed some potentiation but not that equivalent to the sum of their individual water numbers (Table V).

TABLE V.—WATER NUMBERS OF MIXTURES OF FATTY ALCOHOLS

Fatty Alcohols	White Petrolatum Calcd.	White Petrolatum Found	Yellow Petrolatum Calcd.	Yellow Petrolatum Found
Decanol + Lauryl	43.5	20.1	46.9	24.1
+ Myristyl	52.9	27.2	54.4	26.3
+ Cetyl	56.6	30.6	60.0	31.9
+ Stearyl	61.3	35.4	65.8	38.0
Lauryl + Myristyl	60.2	20.1	61.7	22.1
+ Cetyl	64.9	35.4	67.3	38.1
+ Stearyl	68.6	38.8	72.3	40.2
Myristyl + Cetyl	73.3	47.2	74.8	48.8
+ Stearyl	78.0	51.0	79.8	54.5
Cetyl + Stearyl	81.7	63.5	85.4	60.1

The water number of the formulas containing wool fat and a single fatty alcohol-petrolatum (Formula B) was roughly that of the sum of their individual components (Table VI).

The emulsions were all of the water-in-oil type.

This work is to be extended to include the polyhydroxy alcohols and the alcohol derivatives. We intend also to investigate the activity of the alcohols in other fats. The results of these investigations will be reported at a later date.

TABLE VI.—WATER NUMBER OF WOOL FAT, FATTY-ALCOHOL, AND PETROLATUM MIXTURES

	F. A., %	W. F., %	Pet., %	Yellow Petrolatum Calcd.	Yellow Petrolatum Found	White Petrolatum Calcd.	White Petrolatum Found
Decanol[a]	12.5	5[b]	82.5	96.6	64.1	98.3	62.1
Lauryl[a]	9.0	5[b]	87.0	103.9	82.5	105.6	86.3
Myristyl[c]	8.0	10	82.0	113.3	97.1	113.1	93.2
Cetyl[c]	4.0	10	86.0	117.0	108.3	118.7	104.1
Stearyl alcohol[c]	6.0	10	84.0	121.7	114.5	123.7	118.2

[a] An extremely soft preparation resulted. More water may have been taken up but it could not be levigated. It resembled a cream in consistency. The odor of the preparation was objectionable.
[b] A 5% concentration of wool fat in petrolatum has the same water number as a 10% concentration.
[c] A softened preparation resulted by the incorporation of the maximum amount of water to the base. Using 10% less water resulted in a stiffer base.

CONCLUSIONS

1. Of the series studied, cetyl alcohol had the lowest optimum concentration and stearyl alcohol caused the greatest potentiation of the water number of petrolatum.

2. "Sweating" was evidenced in the saturated mixtures and not in the unsaturated mixtures at higher temperature (45°). The results seem to indicate that the water number is influenced by temperature.

3. The water number of a mixture of fatty alcohols is not the sum of the individual water numbers.

4. The water number of a mixture of wool fat-fatty alcohol and petrolatum is "roughly" the sum of the water numbers of the individual components.

5. The emulsions were all of the water-in-oil type.

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Effect of Sodium 5-Allyl-5(Methylbutyl)barbiturate (Sodium Seconal) on Oxygen Consumption in Rats*

By NILKANTH M. PHATAK and EDWARD SAXEY

The results of a study of the effect of subanesthetic doses of Seconal on oxygen consumption are reported. Peoples' photographic method was modified to measure continuous oxygen consumption of rats. In 30 rats the average rate of oxygen consumption under control conditions was 4.5 cc. per minute which was reduced by Seconal to 4.0 cc. per minute with no change in respiratory rate. In 5 rats, not included in the results above, slight excitation was produced so that the oxygen consumption increased from 4.4 cc. per minute to 5.1 cc. per minute without effect on the respiration. These results show that in the majority of animals a significant reduction in oxygen consumption (metabolic rate) occurs with subanesthetic doses of Seconal without change in respiratory rate, or due to the effects of muscular relaxation.

GUEDEL (1) states that the "reflex irritability" of an individual under general anesthesia parallels the metabolic rate, which in turn reflects the oxygen demand of the body cells. Since preanesthetic sedatives contribute an appreciable share of the depression produced in anesthesia, the extent of their effect on oxygen consumption is of importance. The barbiturates may influence oxygen consumption through one or through a combination of the two following mechanisms: (*a*) direct (systemic) depression of intracellular enzymic oxidative processes, or (*b*) indirect cellular anoxia produced subsequent to respiratory center depression. The danger of anoxia is greater when the particular preanesthetic depressant and the size of its dose tend to depress the process of respiration more than the sum-total of vital, intracellular oxygen consumption. A desirable barbiturate thus is one which can afford a wide range, or safety margin, between the dose producing a definite decrease in oxygen consumption rate and that depressing the respiratory function.

Previous studies concerning effects of barbiturates on metabolic rate show contradictory results. Anderson, Chen, and Leake (2) in obtaining evidence for Guedel's metabolic gradient concept, found that Amytal, in therapeutic doses, produced a slight increase in the oxygen consumption rate of normal individuals. This effect, however, was reversed by larger doses. They also noted an increase in the respiratory rate after amytal administration.

Lee (3) injected adult rats subcutaneously and intraperitoneally with gradually increasing doses of Amytal and observed a corresponding decrease in the basal metabolic rate. The route of administration had no influence in his results.

Siebert and Thurston (4) studied effects of subcutaneous injections of Amytal in guinea pigs. They found a decreased rate of heat production in these animals. Kleiber and Saunders (5) injected rats subcutaneously and intraperitoneally with Amytal. They noted that thirty minutes after the intraperitoneal injections, the oxygen consumption rate decreased 68 per cent of that for the controls; and 80 per cent of that for the controls after subcutaneous administration. When the Amytal dose was adequate to produce anesthesia, a considerable decrease in the metabolic rate of the animals was noted.

The effects of barbiturates on metabolism are considerable when given in large doses, but in ordinary doses their action on metabolism is held to be negligible. As Peoples (6) puts it, "the effects of barbiturates on oxygen consumption are no more than would be expected from a similar degree of relaxation in sleep." Other experimental observations of Deuel on dogs (7) and of Rowntree (8) on human subjects also indicate the rather inconclusive effects of barbiturates on oxygen consumption.

Our study is limited to the effects of sub-

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cutaneous administration of subanesthetic doses of Sodium Seconal on the oxygen consumption of rats. This drug is metabolized predominantly through the liver. Since central nervous system depressant drugs produce rather pronounced metabolic effects through sympathetic stimulation (reflex adrenin mediation), we thought it would be of interest to note the effects of this type of short-acting barbiturate on the oxygen requirements of the body.

METHODS

A modification of Peoples' photomotographic method (9) was utilized to obtain visual records of a continuous oxygen consumption rate of young rats of both sexes. All animals served as their own controls since previous to Seconal injections, a record of the animal's oxygen consumption was obtained over a control period of sixty to ninety minutes. During this period the rats were accustomed to the closed oxygen system of the apparatus. By extending the control observations to longer periods it was possible to discount for the periodic contributions to increased oxygen consumption caused by the sporadic activity of the rats. (See Fig. 2.) Summating the results of these prolonged control and observation periods made it possible to obtain reliable "average" rates of oxygen consumption for comparison. (See Figs. 3 and 4.) The glass jar, in which the animal was placed, was immersed in a constant temperature water bath at 28° since the flow meter on the apparatus was calibrated at this arbitrary point. (See Fig. 1.) The temperature of the bath was regulated so as to not vary 0.2°. This procedure eliminated effects of variations of temperature entering into the calculations of the graphic oxygen consumption figures.

RESULTS

The results of observations on 30 rats injected subcutaneously with Sodium Seconal and accompanying data are presented in Table I. For comparison of dosage variation the results are also summarized in Table II.

It is apparent from the results that subanesthetic doses of Sodium Seconal, like other barbiturates, produce variable degrees of central nervous system depression depending on individual variation. Often the same dose produces increase of muscular activity

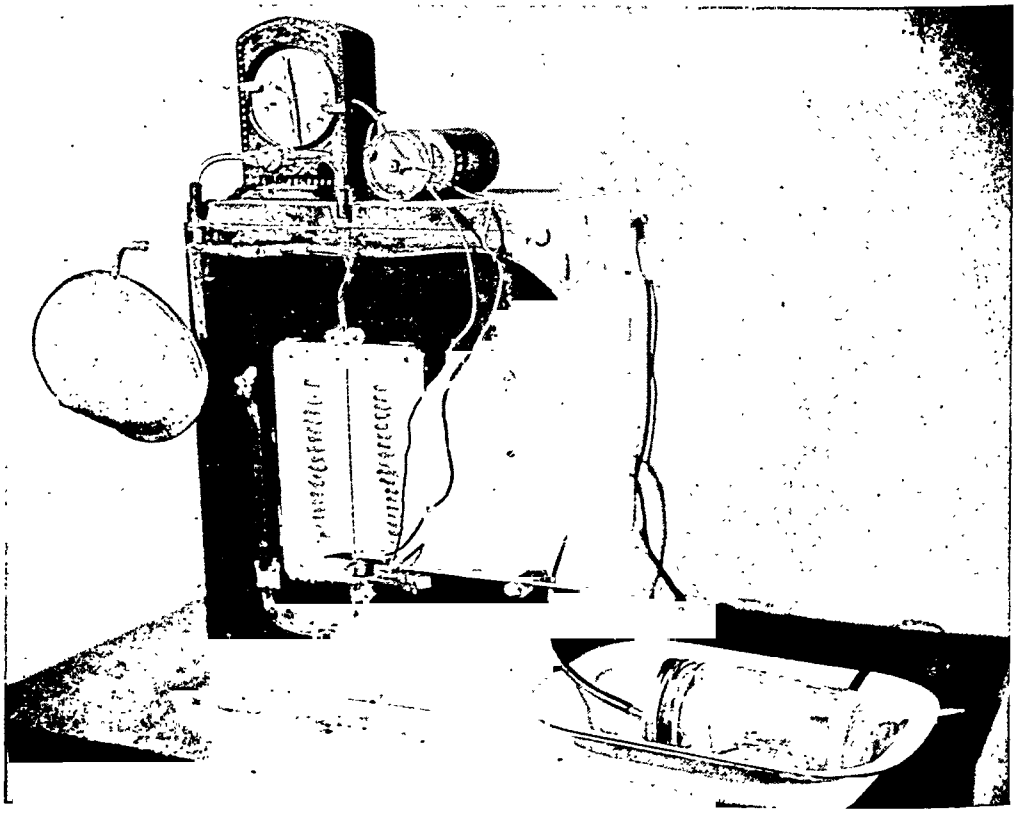

Fig. 1.—Metabolism apparatus. Modified Peoples' photographic metabolar.

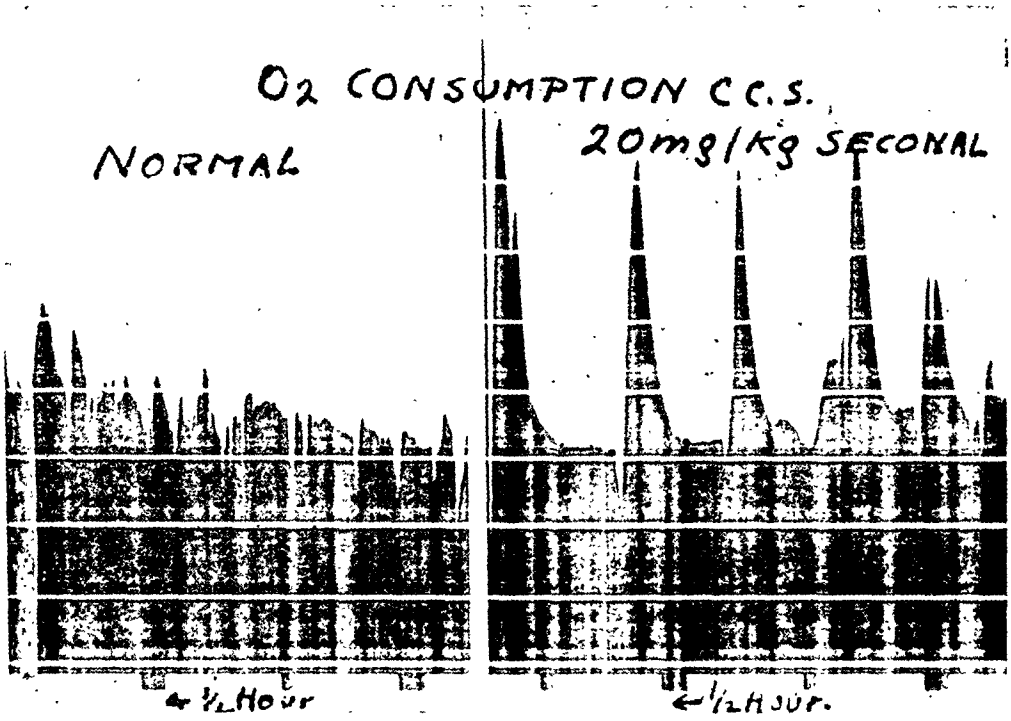

Fig. 2.—Showing periodic spurts of increased activity (sharp peaks on the right half of the record) of rats during the experimental period.

TABLE I.—EFFECT OF "SECONAL SODIUM" INJECTIONS ON O_2 CONSUMPTION RATE IN THIRTY WHITE RATS

Sex	Wt., Gm.	Dose, Mg./Kg.	Pre-Injection Period Time, Min.	Pre-Injection Period Av. O_2 Used Cc./Min.	Post-Injection Period Time, Min.	Post-Injection Period Av. O_2 Used Cc./Min.	Remarks
M	139	20	90	4.0	90	3.5	L.A.[a]
M	140	20	120	4.2	105	4.0	L.A.
M	150	20	120	3.75	180	4.0	Quiet 60 min.; L.A. 120 min.
F	170	20	90	3.0	90	2.5	L.A. throughout
F	160	20	120	4.5	150	4.0	L.A. throughout
F	160	20	90	4.0	120	3.5	L.A. throughout
F	130	20	120	3.0	150	2.5	L.A. throughout
F	162	25	150	6.0	150	5.0	L.A. throughout
F	115	25	90	4.0	210	3.5	Quiet 60 min.; L.A. 150 min.
F	92	25	150	4.0	210	3.5	Good relaxation 30 min.; L.A. 180
F	154	25	150	5.0	150	4.0	Quiet 60 min.; Very L.A. 90 min.
F	110	25	150	3.25	210	2.75	Quiet throughout
F	85	25	150	2.50	150	2.0	Asleep 60 min.; L.A. 90 min.
F[b]	161	25	120	3.75	180	5.0	Excitement throughout
M[b]	146	25	150	3.50	110	4.5	Quiet 30 min.; Mild excit. 90 min.
F	110	25	150	3.5	150	3.25	Quiet 90 min.; L.A. 60 min.
F	123	25	120	3.5	240	3.0	Quiet 60 min.; L.A. 180 min.
F	186	25	120	6.0	150	5.0	L.A. throughout
F[b]	152	25	180	4.5	180	4.5	Mild excitation 90 min.; L.A. 90 min.
F	152	25	150	5.0	150	4.5	L.A. throughout
M[b]	120	25	120	5.25	120	6.0	Mild excitation throughout
F	157	25	120	4.75	120	4.25	Some excitation 60 min.; L.A. 60 min.
F	151	25	90	5.00	90	4.75	L.A. throughout
M	144	25	150	4.75	120	4.50	Quiet 30 min.; L.A. 90 min.
F	141	25	180	4.50	210	4.00	Quiet 30 min.; L.A. 180 min.
M	183	25	180	5.00	180	5.50	Excitation 30 min.; Awake 150 min.
M	180	25	120	5.25	210	5.00	L.A. 90 min.; Awake 120 min.
M	157	25	150	4.75	210	4.50	L.A. throughout
M	195	25	90	5.00	150	4.50	L.A. throughout
F	160	25	120	4.00	180	3.25	L.A. throughout

[a] L.A. = Light Anesthesia.
[b] These animals showed excitation rather than depression.

TABLE II.—COMPARISON OF EFFECTS OF "SECONAL SODIUM" INJECTIONS ON OXYGEN CONSUMPTION RATE IN MALE AND FEMALE RATS SEPARATELY AND IN BOTH GROUPS TOGETHER

No. of Rats and Sex	Dose of Seconal, Mg./Kg.	Average Oxygen Consumption: Pre-Injection, Cc./Min.	Post-Injection, Cc./Min.	Change, Cc./Min.
M— 3	20	4.00	3.83	−0.17
F— 4	20	3.63	3.13	−0.50
7 total	20	3.79	3.43	−0.36
M— 4	25	4.94	4.63	−0.31
F —14	25	4.36	3.77	−0.59
18 total	25	4.49	3.96	−0.53
M— 3	25	4.58	5.33	+0.75
F— 2	25	4.13	4.75	+0.62
5 total	25	4.40	5.10	+0.70
25	25	4.47	4.21	−0.26
30	20 and 25	4.50	4.00	−0.50

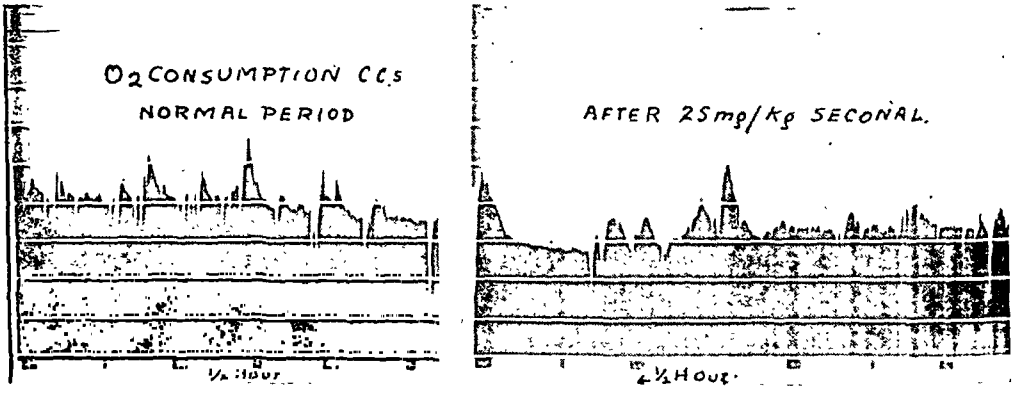

Fig. 3.—Showing that by a prolongation of the recorded observation periods, both for the control and experimental parts, reliable "average" rates of Oxygen Consumption can be obtained. Each black bar at the base represents one-half hour interval of time. The sharp peaks in the records are due to spontaneous periodic increases of activity of the experimental rats.

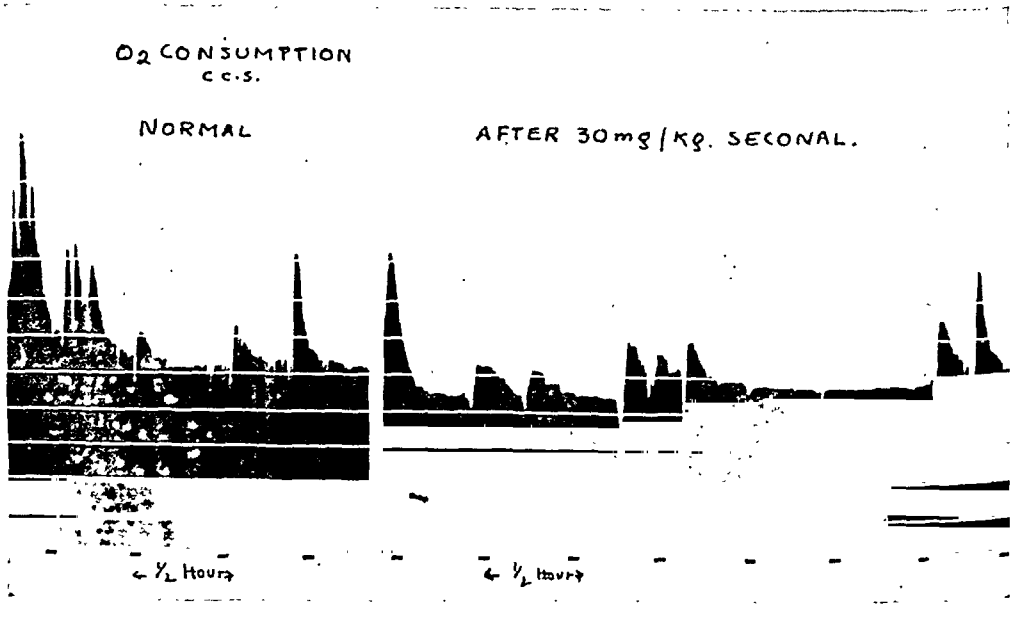

Fig. 4.—(See legend for Figure 3.)

in a few animals and thus increases their oxygen consumption rather than decreases it. We have found no marked changes in respiratory rates beyond those produced by the tranquilizing effect of the barbiturate. The rats showed sedation and light anesthetic effects of the drug. They could be easily aroused to activity by mechanical stimulation and they retained a good degree of skeletal muscle tone at the dosage levels indicated in Table I. The averages of the rate of O_2-consumption per minute of the various groups of animals are shown separately in Table II. In 5 rats included in this series, where Seconal produced slight excitation, the average O_2-consumption rate increased from a normal of 4.4 cc. to 5.10 cc. per minute. There is a greater degree of depression of oxygen consumption in the female rats than in the males.

CONCLUSIONS

In conclusion, the results indicate that in the majority of rats a significant reduction in oxygen consumption (metabolic rate) occurs when subanesthetic doses of Sodium Seconal are injected subcutaneously. In this paper the mechanism of the depressant effects of Seconal on oxygen consumption is not investigated. It also appears that Seconal, in the doses used, did not produce any changes in the respiratory rate and that muscular relaxation was not a contributory factor in the reduction of oxygen consumption under our experimental conditions.

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The Lipolytic Activity of Pancreatin U. S. P.*

By CHARLES W. BAUER† and EDWIN E. WILSON‡

A method of analysis which can be used successfully to determine the lipolytic activity of Pancreatin, U. S. P., is presented.

PANCREATIN U. S. P. contains the enzymes steapsin, amylopsin, and trypsin. The U. S. P. gives the minimum standards required for amylopsin and trypsin, but gives no minimum lipolytic activity. The only pharmacopœia requiring such standards is the British Pharmacopœia (4) which uses "the cream of fresh milk" as a substrate.

It appears that the reasons for not giving the lipase activity of Pancreatin, U. S. P. are the following:

(*a*) the inability to obtain a preparation with a large amount of lipase;
(*b*) the lack of a good assay; and
(*c*) the labile character of lipase.

The object of this investigation is to determine the lipolytic activity of Pancreatin U. S. P. and to find a method of assay whereby uniform results may be obtained. The degree of activity which is to be determined will not be based upon physiological conditions. This determination is impossible, since neither the length of time of the action of lipase in the animal body nor the influence of all the physiological activators or inhibitors has as yet been determined. For the same reasons minimum requirements given for trypsin and amylopsin in Pancreatin U. S. P. are not based upon the physiological action of these substances, and should not be so interpreted. It should be clearly understood that the degree of activity is merely determined under specific conditions suitable for analytical purposes, which may have little relationship to physiological conditions.

The literature of the biochemical journals gives numerous methods for the assay of lipase. A number of the better-known

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‡ This paper is based on a thesis presented to the Graduate Council of the Massachusetts College of Pharmacy by Edwin E. Wilson, in partial fulfillment of the requirements for the degree of Master of Science in Pharmacy.

methods of determination were tried and abandoned. The assay formulated by Willstätter, Waldschmidt-Leitz, and Memmen (1), with certain modifications, was most satisfactory for this investigation of U. S. P. Pancreatic lipolytic activity.

EXPERIMENTAL ANALYSIS

The Lipolytic Activity of Pancreatin U. S. P.—The determination of pancreatic lipase, as formulated by Willstätter, Waldschmidt-Leitz, and Memmen (1), is performed by titrating the liberated fatty acids produced as a result of the action of the fat-hydrolyzing enzyme steapsin, upon the substrate, olive oil, after digestion at 30° for one hour. The digestion mixture contains the enzyme preparation, ammonia-ammonium chloride buffer, calcium chloride, albumin, and olive oil. This reaction mixture is titrated in an alcoholic medium with 0.1 *N* alcoholic potassium hydroxide, using thymolphthalein as the indicator. The amount of 0.1 *N* alkali used, corrected for control, is the amount required by the liberated fatty acids. Willstätter and co-workers then converted the amount of alkali used into per cent of fat split. The per cent of fat split was then translated into lipase units. One Lipase Unit is the amount of enzyme required under the conditions specified above to split 24% of 2.5 Gm. of olive oil of a saponification value of 185.

If the splitting is more than 24%, the experiment should be repeated with one-half as much of the enzyme as was used before. If the splitting is less than 10%, the experiment should be repeated with twice as much enzyme. The percentage must be not less than 10% nor more than 24%; otherwise the lipase units cannot be determined. These limits have been incorrectly reported in many standard textbooks (3).

The Accuracy of the Assay Method of Willstätter, Waldschmidt-Leitz, and Memmem.—The digestion mixture of Willstätter, composed of several water-insoluble substances, fails to give a reaction that is uniform for the entire period of digestion. The presence of a water-insoluble substrate would seem to militate against uniform digestion, and this assumption was confirmed experimentally. Upon repetition of the experiment, a series of divergent readings was obtained. The average titration value was 12.76 cc. The standard variation computed from these results was 1.12 cc. The presence of this sizable variation suggested the search for a satisfactory inert emulsifying agent which could be used to get more nearly uniform reactions and consequently more nearly comparable titration values.

The Value of Bentonite in the Analysis of U. S. P. Pancreatic Lipolytic Activity.—After a search for inert emulsifying agents which would give more nearly homogeneous reaction mixtures, bentonite was found to be the most satisfactory substance tried. The use of a blank determination ruled out any effects that bentonite might have upon the reagents which could possibly lead to an erroneous interpretation of enzymatic activity.

Three cubic centimeters of Magma of Bentonite N. F. was used in place of the 3 cc. of water used by Willstätter. The use of bentonite reduced the standard variation from 1.12 cc. to 0.27 cc.

The Amount of Pancreatin U. S. P. That Can Be Used to Determine Its Lipolytic Activity by the Willstätter Method in the Presence of Bentonite.—It was found that more than 400 mg. of Pancreatin U. S. P. in the presence of bentonite failed to give uniform results. This was also true in the absence of bentonite. Any amount between 200 and 400 mg. of Pancreatin U. S. P. analyzed gave results from which a satisfactory and uniform curve could be plotted.

The Effect of Bile Salts on the Titration Values in the Presence of Bentonite.—Bile salts produced a significant lipase activation when added to the digestion mixture at the start, if the medium was alkaline; but the yellow color imparted to the reaction mixture, because of the bile salts, made it very difficult to get a sharp end point. Since a sufficient degree of lipase activity is obtained without the use of bile salts, and since the end point is more readily determined in the absence of them, it was deemed advisable to determine U. S. P. lipase activity without making further use of this impure activator.

The Abandonment of Albumin as an Activator.—The influence of albumin on lipase activity is believed to be something more than that of activation. Willstätter used albumin to compensate for the inhibitory effect of certain unidentified substances found in pancreatic preparations. The use of albumin for this purpose is subject to criticism. In the first place, it is difficult to get two samples of albumin of identical composition. In the second place, Pancreatin U. S. P. contains a proteolytic enzyme which acts upon albumin to form acids which influence the titration value materially (2). Since the lipase activity is based upon the amount of alkali required to neutralize the acids produced as a result of lipolytic activity, every precaution must be taken to avoid the formation of acids in any other way.

The titration values of lipase activity were sufficiently high in the absence of albumin to warrant its elimination, and the acidity so obtained is a true index of the amount of fatty acids produced as a result of pancreatic lipolytic activity.

The standard variation for the titration values in the presence of albumin and bentonite was found to be 0.27 cc. In the absence of albumin but in the presence of bentonite it was found to be 0.19 cc.

The Effect of Various Amounts of Bentonite on Lipolytic Activity of Pancreatin U. S. P. in the Absence of Albumin.—It appears that from 3 to 5 cc. of Magma of Bentonite N. F. produces the best results for analysis in the absence of albumin, and that this amount of bentonite has no appreciable effect upon the enzyme. The chief value of the bentonite is the production of a uniform mixture, by

means of which more of the fat is brought into contact with the enzyme, and which consequently gives a higher titration value in a given period of time.

The Amounts of Pancreatin U. S. P. That Can Be Used to Determine Its Lipolytic Activity in the Absence of Albumin but in the Presence of Bentonite.—It was found that more than 400 milligrams of a sample of Pancreatin U. S. P. could not be used if the lipase content were to be determined by the Willstätter Method. It was thought to be advisable to determine the amount of Pancreatin U. S. P. that could be assayed by this method in the absence of albumin but in the presence of bentonite. Here, too, it was found that 400 mg. was the upper limit that could be used to make this determination.

A Comparison of the Different Methods Used to Determine the Lipolytic Activity of a Sample of Pancreatin U. S. P.—Bentonite favors the formation of a finely divided substrate, giving a higher degree of digestion and more nearly uniform results. It appears that albumin either activates the lipase, neutralizes the action of some inhibitory substance, or serves as a substrate for the proteolytic enzymes in pancreatin. The increase in acidity may be due to either one or all of these three actions of albumin. A comparison of the data obtained by different methods of assay is shown in Table I.

TABLE I.—A COMPARISON OF PANCREATIC LIPOLYTIC ACTIVITY OF DIFFERENT METHODS OF ASSAY

Method of Analysis Used	Cc. of 0.1 *N* Alcoholic Potassium Hydroxide
	10.66
Willstätter	10.91
	8.31
	20.81
Willstätter (with bentonite)	20.00
	20.00
	17.36
Willstätter (with bentonite but without albumin)	17.21
	17.84

The Influence of Ether on the Lipolytic Activity of Pancreatin U. S. P.—In a series of experiments, 10 Gm. of Pancreatin U. S. P. was shaken intermittently with 500 cc. of ether for twelve hours at room temperature. The suspension was filtered and both the filtrate and residue were assayed. The ether-soluble fraction showed no lipolytic activity.

The ether insoluble fraction showed the same degree of lipolytic activity as that of an equal amount which had not been treated with ether. These results indicate that pancreatic lipase is neither soluble nor is it destroyed to an appreciable extent by ether. This finding leads one to question the use of ether for the purpose of stopping lipolytic activity as is done in the Willstätter Assay. The data of these findings are shown in Table II, and contradict the literature on the subject (3), which states that ether stops the action of lipase.

In a series of digestions, carried out as in previous experiments, the digestion mixture was washed with alcohol into a titration flask containing 20 cc. of ether. The mixture was made up to a volume of about 125 cc. with alcohol, and was titrated with 0.1 *N* alcoholic potassium hydroxide. In the control flasks, numbers 4, 5, and 6, (Table III), the pancreatin was added just before the titration. Flasks 1, 2, and 3 were titrated one hour after adding the pancreatin and were then allowed to sit for forty-eight hours at room temperature and again titrated. At the end of forty-eight hours additional potassium hydroxide was required to neutralize the acid which had been formed during this time. Flasks 7 and 8 were not titrated until they had sat for forty-eight hours at room temperature.

TABLE II.—THE LIPASE ACTIVITY OF THE ETHER INSOLUBLE FRACTION OF PANCREATIN U. S. P.

Sample Analyzed	Mg. of Pancreatin Used	Cc. of 0.1 *N* Potassium Hydroxide
Untreated sample	300	16.07
	300	16.07
Ether insoluble fraction	300	15.06
	300	14.35

From these titration values it may be seen that the ether does not destroy the lipase activity of Pancreatin U. S. P. If, on the contrary, ether destroyed the lipase action then the readings should have been no greater for flasks 7 and 8 than the total titration values given for numbers 1, 2, and 3. These data suggest that the retarding effect in the first three flasks were due to either the alcoholic potassium hydroxide or the *p*H of the solution. It is obvious from these findings that ether may retard, but it does not destroy, the lipolytic activity of Pancreatin U. S. P.

TABLE III.—THE EFFECT OF ETHER ON LIPOLYTIC ACTIVITY

Flask No.	Mg. of Pancreatin	Cc. of Alkali after 1 Hr. Digestion	Cc. of Alkali 48 Hr. after Adding Ether	Total Cc. of 0.1 *N* Potassium Hydroxide
1	300	54.6	3.2	57.8
2	300	55.0	5.1	60.1
3	300	54.3	3.2	57.5
4 (control)	300	33.4	1.2	34.6
5 (control)	300	33.9	1.5	35.4
6 (control)	300	34.1	1.5	35.6
7	300	..	84.1	84.1
8	300	..	82.5	82.5

The Lipolytic Activity of Pancreatin U. S. P. in the Absence of a Buffer, but in the Presence of Bentonite.—Since bentonite was found to be an efficient emulsifying agent whose action as a buffer has not been tested, it was felt that it might serve a double purpose and that the ammonia-ammonium chloride buffer might advantageously be eliminated. However, the titration values obtained in the absence of the ammonia-ammonium chloride buffer were too low for advantageous elimination.

An Improved Method of Analysis for the Determination of Pancreatic Lipase.—Into a glass-stoppered 50-cc. Erlenmeyer flask, introduce exactly 2.5 Gm. of Olive Oil U. S. P., 3 cc. of Magma of Bentonite N. F., 0.5 cc of 2% calcium chloride solution, and 2 cc. of buffer (0.66 cc. of normal ammonium hydroxide and 1.34 cc. of normal ammonium chloride). Rotate this mixture several times so as to produce a thick emulsion. Make the enzyme preparation containing 300 mg. of pancreatin, up to 10 cc. with distilled water; add this preparation to the flask and agitate vigorously for one minute so as to emulsify thoroughly the mixture. Place this reaction mixture in a constant temperature bath adjusted to 30°. Exactly one hour after the introduction of the enzyme preparation, remove the flasks and the controls, wash their contents with Alcohol U. S. P. into larger flasks containing 20 cc. of ether so as to make a total volume of 125 cc. Add twelve drops of 1% thymolphthalein and titrate immediately with 0.1 *N* alcoholic potassium hydroxide.

Do not add the enzyme preparation to the control flasks until they are removed and ready for titration. Subtract the titration of the control determination from the enzyme-digestion mixture in order to get the amount of alkali that is used to neutralize the fatty acids as a result of lipolytic activity.

The Lipase Activity of Commercial Samples of Pancreatin U. S. P.—To determine the lipase content of commercial samples of pancreatin, samples were obtained from different drugstores. These preparations were manufactured by three different pharmaceutical houses from whom was ascertained the exact age of each sample. These samples had been opened and stored under various conditions. Some samples were powdery, light-cream-colored preparations, while others were caked and were dark brown in appearance. These samples were assayed by the method given above. The analyses indicate that the age of the sample is not the factor most significant of its lipolytic activity. The data in Table IV show that some of the older samples contained almost as much lipase as did the more recent.

TABLE IV.—THE LIPASE ACTIVITY OF 300-MG. SAMPLES OF PANCREATIN COMING FROM VARIOUS MANUFACTURERS

Sample No.	Company	Age in Mo.	Cc. of 0.1 *N* Potassium Hydroxide
1	A	15	17.966
2	B	15	26.455
3	B	13	27.800
4	C	72	23.075
5	C	36	24.000
6	B	180	8.700
7	B	>180	1.050

The analyses seem to indicate that Pancreatin U. S. P. contains enough steapsin to be included in the minimum requirements of the official definition. An official method of assay similar to the one used for these assays could then be adopted.

RECOMMENDATION

The data obtained in this investigation suggest the possibility of introducing the following paragraph into the official definition of Pancreatin U. S. P. (5).

The potency of Pancreatin U. S. P. shall be such that when assayed as directed it shall convert not less than its own weight of Olive Oil U. S. P. into fatty acids in one hour.

The adoption of this paragraph would also require the introduction of an assay method, such as has been recorded in this thesis, into the official monograph of Pancreatin U. S. P.

SUMMARY

1. Pancreatin U. S. P. has a measurable amount of lipolytic activity according to Willstätter's method of analysis.

2. The results of the assay of Pancreatin will vary in accordance with its age and method of analysis, and probably with the method of manufacture and storage.

3. Bentonite is an inert emulsifying agent which is very useful in the determination of lipolytic activity.

4. Ether appears to have no effect upon lipolytic activity and may not be used to stop the reaction. However, the ether should be retained in the assay, because it dissolves the undigested olive oil and hence gives a clearer end point.

5. The color produced by bile salt preparations makes them undesirable activators in this determination.

6. Albumin should not be used in this assay since it is acted upon by the proteolytic enzymes present in the pancreatin.

7. A method of analysis has been worked out which can be used successfully to determine the lipolytic activity of Pancreatin U. S. P.

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A Critical Investigation of the Amylolytic Activity of Pancreatin and Extract of Malt*

By CHARLES W. BAUER† and ALGIRD JOHN RASKAUSKAS‡

The age of the product does not determine its amylolytic activity. A good appearing pancreatin eleven years old had a higher amylase activity than an off-colored, but recently manufactured, product. The official definitions of pancreatin and extract of malt are misleading. The official definitions should give the time involved in the assays in order to give a truer picture of their enzymatic activities.

KIRCHHOFF (in 1814) was the first to record the effect of a biochemical catalyst when he observed the influence of the glutinous component of wheat meal upon the conversion of starch to soluble carbohydrates. This phenomenon was later classified as enzymatic activity by Kühne (1878). A great deal of work has been done in determining amylolytic activity of substances since that time, yet the official method recorded in the United States Pharmacopœia for the amylase activity of Pancreatin and Extract of Malt is still subject to considerable criticism.

In order to evaluate the official assay method now in use a critical investigation of the substrate and its preparation, of the period of digestion, of the end point, of the temperature, and even of the official definition should be made. Starch, without indicating its source, is the conventional substrate used in most qualitative and quantitative assays. The Pharmacopœia specifies potato starch. Since the chemistry of the starches is not entirely understood, and since there are individual differences which manifest themselves in digestion experiments, the choice of a particular starch in the Pharmacopœia may be looked upon as worth while. The use of potato starch is based upon the findings of Graber (1). The theory that starches are made up of different components in varying proportions, some of which are attacked by one group of amylases and some by others, makes the choice of the substrate even more important. Without specifying any botanical variety, Van Klinkenberg (2) asserts that starch is a mixture of 36 per cent α-starch and 64 per cent β-starch. Kühn (3) does not subscribe to the "mixture theory." He believes that starch is a single molecule with a chain of alternating α and β glucosidic linkages. Here again, the nature of the amylase will determine whether it will attack the α or the β linkage which will materially influence the nature of the intermediary products.

It has been fairly well established that starch consists of two substances, α-amylose or amylopectin, found in the outer layer of the granules and not readily dispersible in water, and β-amylose found in the interior and dispersible in water. Various workers have separated these two components and have reported yields of soluble amylose varying from 17 per cent to 98 per cent depending upon the method of separation. According to Taylor and Iddles (4) the yield of α-amylose depends upon the botanical source of the starch. Potato starch gave the lowest yield of those varieties tried. This may explain why potato starch, according to Graber (1), is more readily converted into soluble carbohydrates by pancreatic amylase than are the other starches.

Kühn (3) made the important observation that pancreatic and salivary amylases act upon starch to give primarily α-maltose. He classified these enzymes as the *alpha* type. He named another group of amylases, obtained from sprouted and unsprouted grains, the *beta* amylases, since they act upon the same substrate to give a large yield of β-maltose.

Various methods are used for the estimation of amylolytic activity. Chief among these are:

1. Measurement of the reducing sugar

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† Associate Professor of Chemistry, Massachusetts College of Pharmacy.
‡ This paper is based on a thesis presented to the Graduate Council of the Massachusetts College of Pharmacy by Algird John Raskauskas, in partial fulfillment of the requirements for the degree of Master of Science in Pharmacy.

formed as a result of the digestion of starch by an amylase.

2. Measurements of the decrease in viscosity of the reaction mixtures.

3. Observations of the changes in color given by the reaction mixtures when treated with iodine.

4. Determination of residual amyloses.

The United States Pharmacopœia recognizes the third method for the quantitative determination of amylase in Pancreatin and Extract of Malt. An important objection to this method of analysis is that the assay methods use different intervals of time for the digestion period. Although the interval of time is given in each of the respective assays, it is not indicated in the official definition of either. The quantitative values given in the official assays are indeed misleading.

The object of this investigation is to find a more satisfactory method of analysis for amylase activity than that given by the United States Pharmacopœia, and to suggest changes in the monographs for Pancreatin and Extract of Malt which will avoid the misinterpretations which are now commonly made. The present monographs lead many to believe that Pancreatin contains about five times as much amylase as Extract of Malt, but the true ratio is about 30 to 1.

EXPERIMENTAL

The potato starch paste made by the official method either under Pancreatin or Extract of Malt is a heterogeneous mixture which does not give uniform digestion. The color of the end point of the assay is very difficult to read accurately, and the error is thus magnified. Furthermore, no consideration is given to the influence of such factors as activators or the control of hydrogen ion concentration.

It was found that a more nearly homogeneous starch paste could be obtained if, after being boiled, it was run through a hand homogenizer several times. The homogenized paste is less viscous than the official paste. A small amount of starch is lost in the homogenizing, which can be corrected by adding 1% of potato starch to the original amount. If this is done the loss is negligible (about 0.1%).

An alternate method is to make a paste by boiling 5.625 Gm. of dry starch for five minutes with 125 cc. of distilled water in a tared beaker of 250 cc. capacity. It is cooled to room temperature and enough distilled water is added to make the mixture weigh 150 Gm. One hundred grams of this paste (containing 3.175 Gm. of dry starch) is used.

In the official assay of amylase activity on potato starch, the end of the reaction is indicated by the absence of a blue or violet color between the iodine and the starch digestion mixture. The starch digestion mixture reacts with iodine to give colors which vary from blue to violet, to various shades of red and pink; and as digestion proceeds they finally fade to the color of the standard iodine solution which is employed. The temperature of the iodine solution must be carefully controlled, since varying the temperature not only affects the time required to reach the official end point, but gives color variations peculiar to the temperature employed.

The assays were run according to the directions given in the United States Pharmacopœia with different temperatures of the iodine solution in order to determine the influence of this factor.

It was found that the temperature of the iodine test solution plays an important role in determining the end point. The tests were made at fifteen-second intervals.

The standard used was the blue solution obtained by adding 0.1 cc. of the digestion mixture (0.15 Gm. of pancreatin added to 3.75 Gm. of dry starch made into a paste and digested at 40° for five minutes) to the dilute iodine solution maintained at 24.8°.

At 12° the starch iodine solution showed a distinctly bluish green color which tended to go to blue. At 25° the solution was a decided blue, with a tendency toward purple as the digestion continued. At 38° the solution, even during the first few minutes of digestion, showed a red color which gradually faded.

Table I shows the digestion time that is necessary in order to get the color with iodine that matches the standard.

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2	40	15.3	6.0
3	40	18.6	5.75
4	40	24.8	5.0
5	40	32.0	3.5
6	40	38.5	1.5

The United States Pharmacopœia states that the temperature of the iodine test solution should be "23° to 25°." It is apparent that this temperature is important and must be maintained for comparative work, for any variations in the temperature of the iodine solution will materially influence the results.

It was found to be very difficult to match the colors considered to be the true end point. However, the intensity of the colors could be compared satisfactorily.

The sensitivity of the homogenized starch iodine test was determined as follows: 1.0 Gm. of dry starch, accurately weighed, was made into a paste by boiling with 100 cc. of distilled water for five minutes. The paste was cooled to room temperature and homogenized, and distilled water was added to make 250 cc. Various dilutions of this preparation were made and treated with the dilute iodine solution. The tests were made at room temperature (23°). The dilute iodine solution was made according to the Pharmacopœia and consisted of 0.2 cc. of 0.1 *N* iodine in 60 cc. of distilled water.

While the starch iodine reaction could be detected in dilutions of 1 to 20,000 if observed instantly at the point of contact, it could not be detected when the two solutions were mixed as directed to be done in the Pharmacopœia. In fact, 1 part of starch in 2000 parts failed to give a positive test.

Thus a considerable quantity of starch may be undigested and still fail to manifest itself by the official method of identification.

Starch which had not been homogenized was also used. The difficulty of getting uniform dilutions and even distribution of the color produced made it impossible to record comparative values.

The Effect of Light on the Starch-Iodine Color.—Willson (5) suggested that a reference sample of U. S. P. Pancreatin be chosen as a standard, and that the assays of unknown pancreatin preparations be compared with it. It is important to note the influence of light upon any color standard of this character.

A typical blue color of the starch-iodine reaction was obtained by adding a small portion of a partially digested starch paste to the dilute iodine solution. This blue solution was divided into three portions.

Portion A was placed directly in the sunlight. Portion B was kept in the room away from the direct rays of the sun but in diffused light. Portion C was kept in a dark locker when it was not employed as the standard.

A comparison of portion C at the end of two hours with portion A at the end of the same period of time proved clearly that the color fades very greatly in direct sun light. This is true to a lesser extent in diffused light, yet it is significant in quantitative determinations, and indicates that the standard should not be made too far in advance.

Determination of a Satisfactory End Point.—Sherman, Kendall, and Clark (6) determined the end point of starch digestion by using larger amounts of digestion mixture and iodine in a smaller volume than in the official assay. This intensifies the color before the end point is reached and makes the end point, where there is "no color which can be distinguished from that of the untreated iodine solution," more definite.

Experiments were made to determine if this end point could be used in the official assay of Pancreatin with the amounts of digestion mixture and iodine solution called for by the Pharmacopœia.

The use of larger quantities of starch than directed in the Pharmacopœia was also tried to determine its value over a wide range of concentrations. Each succeeding assay contained 3.75 Gm. of additional dry starch diluted in the same ratio as before, with an increase in weight of 100 Gm. over that used in the preceding assay.

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No.	Mg. Pancreatin	Gm. Dry Starch	Total Wt. of Mixture, Gm.	Time, in Min., Required for Digestion
1	150	3.75	100	8.
2	150	7.50	200	20
3	150	11.25	300	36
4	150	15.00	400	50
5	150	18.75	500	61
6	150	22.50	600	75
7	150	26.25	700	101
8	150	30.00	800	109

It will be recalled that in the official method the end point is considered reached when no blue or violet color is produced, but the allowance of some color makes it very indefinite.

It takes the digestion mixture a longer period of time to attain the end point of Sherman, Kendall, and Clark (6) than that given in the Pharmacopœia for its end point. However, it was found that the eight minutes required to reach the end point by this method is comparable to the amount of Pancreatin which takes five minutes by the method of the United States Pharmacopœia, and is much easier to see.

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Instead of 3.75 Gm. directed in the official assay, 3.788 Gm. of homogenized potato starch was used.

The total weight of the mixture was increased from 100 to 150 Gm. to allow for the rinsing of the homogenizer.

Water, previously heated to boiling, instead of the temperature of 50° to 60° was found to hasten the gelatinization of the starch and to prevent the formation of a tough paste at the bottom of the beaker. This paste is not only difficult to homogenize, but is not readily digestible.

The modified end point of Sherman, Kendall, and Clark (6) was employed. The italicized portion of the following monograph indicates the extent to which the official assay was modified.

"Determine the percentage of moisture in potato starch by drying 0.5 Gm. accurately weighed at 120° for four hours. Thoroughly mix a quantity of the starch, equivalent to *3.788 Gm. of dry starch (3.75 Gm. plus 1%)* with 10 cc. of cold distilled water. Add the mixture with constant stirring to 75 cc. of distilled water, *previously heated to boiling*, contained in a 250 cc. beaker. Rinse the remaining starch into the beaker with 10 cc. of distilled water, and boil it gently for five minutes or until a translucent uniform paste is formed. Cool to room temperature. *Run it through a hand homogenizer three times, collecting it in a tared 250 cc. beaker. Pour several 20 cc. portions of hot distilled water into the homogenizer, in order to remove the remainder of the starch paste, and add the rinsings to the paste in the beaker. Add enough distilled water to make the mixture weigh 150 Gm.*

"Warm the paste to 40° and place the beaker in a water bath maintained at 40°. Suspend 0.15 Gm. of pancreatin in 5 cc. of distilled water and add the suspension to the starch paste, mixing it well by pouring the mixture from beaker to beaker for 30 seconds, noting the time when the pancreatin suspension was added to the starch. Maintain the mixture at 40° for exactly *eight* minutes. At once add 0.1 cc. of the starch pancreatin mixture into a previously made mixture of 0.2 cc. of 0.1 *N* iodine in 60 cc. of distilled water. *When viewed against a white background, there should be no color which can be distinguished from that of the untreated iodine solution.*"

The Preparation of β-Amylose.—Sherman and Baker (7) found that pancreatic amylase produced sugar more rapidly from β-amylose than from α-amylose. They also found that in the early stages of digestion malt amylase showed a greater yield from α-amylose than from β-amylose.

In order to see if any appreciable difference in the activity of pancreatic amylase could be obtained by using β-amylose in place of whole starch, a method of obtaining this component was needed. The literature gives various methods which are employed for the separation of the components of potato starch.

Gruzewska (8) effected a separation by treating potato starch with dilute alkali, and after neutralization and sedimentation, decanting the dissolved amylose from the residue of amylopectin. The amylose represented 40–45% of the original starch.

By treatment of potato starch with hot water, sedimentation, and decantation, Tanret (9) estimated 27% of amylose.

Sherman and Baker (7) subjected a thin paste of potato starch to centrifugal force, and obtained about 15% in *beta* amylose form.

Samec and Mayer (10) *electrodiclyzed* a dispersion of potato starch prepared at 120°. The amylose fraction represented 17%.

Ling and Nanji (11) *fractionated* potato starch by freezing a paste, and after melting the frozen mass, separated the solution of amylose from the amylopectin by centrifuging.

Taylor and Iddles (4) disintegrated starch grains, previously treated with hydrogen chloride in alcohol, with ammonium thiocyanate solution, then precipitating with alcohol and subjecting the precipitate to ultra-filtration or dialysis. The yield of soluble amylose was 97–98%.

Baldwin (12) effected separation by gelatinizing, freezing, and performing a series of extractions.

A rapid and satisfactory method was found which consisted of boiling a 2.5% starch paste for thirty minutes to rupture the granules. The paste was then precipitated with alcohol, dried, and powdered. The soluble amylose was separated from the insoluble component by allowing to stand in cold water, decanting, and boiling the aqueous solution to concentrate the β-amylose. The insoluble α-amylose was washed free of β-amylose, precipitated, dried, and powdered. A yield of about 36.5% of β-amylose was obtained.

A Detailed Method of Separating Potato Starch into Alpha and Beta Amylose.—Thoroughly mix a quantity of starch equivalent to 10.0 Gm. of dry starch U. S. P. with 50 cc. of cold distilled water, and add, with constant stirring, to a sufficient amount of boiling distilled water to make 350 cc. Rinse the remaining starch into the beaker with two 25-cc. portions of distilled water and boil gently with constant stirring for thirty minutes. Add distilled water when necessary to maintain the original volume. Allow the paste to cool to room temperature. Transfer the paste to a 2000-cc. Erlenmeyer flask, using 50 cc. of distilled water to remove the remainder of the paste from the beaker. Now superimpose over the starch paste 500 cc. of 95% alcohol, stopper the flask and shake vigorously until the starch is completely precipitated. Filter and allow the precipitate to dry for twelve hours at 65°. Grind the dry altered starch in a mortar, and pass the powder through a number 100 sieve.

Weigh about 5 Gm. of the altered starch and sprinkle it with constant stirring into 500 cc. of cold distilled water in a 2000-cc. beaker. Add enough water to this mixture to make the final volume measure about 1000 cc. and let it settle for twenty-four hours. Decant the clear supernatant liquid and concentrate it by boiling to a volume of about 200 cc. or until the β-amylose will be precipitated with an equal volume of alcohol. Precipitate the β-amylose with 200 cc. of 95% alcohol and dry it at

65°. Powder the precipitate so that it will pass through a number 100 sieve. This was used as the β-amylose in succeeding work.

Saccharogenic Activity of Pancreatin Using Different Substrates.—In addition to β-amylose, potato starch, corn starch, and soluble starch were used.

The method of analysis used was based on the amount of maltose produced from a given amount of substrate in a given unit of time. It is essentially the method of Willstätter, Waldschmidt-Leitz, and Hesse (13). These authors used soluble starch as the substrate. The different substrates being tested were prepared by making one per cent pastes and boiling for five minutes.

It was found that the different substrates gave no appreciable difference in the amount of maltose produced. There appears to be no advantage in using β-amylose for this determination. If any conclusions could be drawn from these findings, the chief one would be that β-amylose is slightly inferior to the other substrates used. The experimental data are recorded in Table III.

TABLE III.—SACCHAROGENIC ACTIVITY OF PANCREATIN USING VARIOUS STARCH SUBSTRATES

Substrate	Mg. Pancreatin	Cc. 0.1 *N* Iodine	Mg. Maltose
Homogenized potato starch	5	4.09	70.14
Potato Starch	5	4.15	71.17
Corn Starch	5	4.27	73.23
Soluble Starch	5	4.07	69.80
β-Amylose	5	3.40	58.31

The Amylase Activity of Different Samples of Pancreatin.—During the course of the experiments it was noted that many of the older samples of pancreatin had caked and had assumed a darker color than usual. Experiments were made to compare the amylolytic activity of the older samples with several fairly fresh samples of pancreatin.

In order to determine accurately the loss in amylolytic activity due to aging, it would be necessary to store a sample of pancreatin under a certain set of conditions, and to determine its activity from time to time. Since this was impractical, several commercial samples of pancreatin were obtained from different drugstores in the vicinity. These samples varied in age from eleven months to fifteen years and came from three different pharmaceutical houses. The conditions under which the samples were stored no doubt varied considerably, since some of the samples were typical cream-colored powders, while other samples were caked, and of a deep brown color.

The activities of these samples of pancreatin were tested by the method of Willstätter, Waldschmidt-Leitz, and Hesse (13). The only change from the original assay was that a 1% homogenized potato starch paste, which had been boiled for five minutes, was used in place of the soluble starch suggested by the authors.

One hundred milligrams of pancreatin was suspended in 100 cc. of distilled water, and 5 cc. of this suspension was added to the substrate.

The pancreatin assayed by the above method showed an irregular drop in amylase activity as the ages of the samples increased. These findings fail to establish any reasons for the irregularity in amylase content. It appears that the age of the sample is of minor consequence, since properly stored samples, judged by their physical appearance, of 16, 53, 60 106, and 132 months of age had practically the same enzyme activity. The samples which were caked and brown in color gave a lower amylolytic activity. Whether these samples were inferior to begin with or changed in appearance and amylolytic activity because of improper storage could not be determined. Nevertheless, it appears that Pancreatin which has caked or is dark brown in color does not have the amylolytic activity which is found in the dry, powdery, light-cream-colored samples.

The experimental work is tabulated to show the source of pancreatin, as derived from companies "A," "B," and "C," and may be seen in Table IV.

TABLE IV.—DETERMINATION OF THE AMYLASE ACTIVITY OF SAMPLES OF PANCREATIN FROM DIFFERENT COMPANIES

Co.	No.	Age Mo.	Cc. 0.1 *N* Iodine	Mg. Maltose
A	1	39	4.53	77.69
A	2	53	4.68	80.26
A	3	92	4.58	78.55
A	4	98	5.58	78.55
B	5	60	4.47	81.29
B	6	60	4.74	81.29
B	7	96	4.69	80 43
B	8	120	3.14	53.85
B	9	132	4.64	79.58
B	10	156	4.02	68.94
C	11	15	3.55	60.88
C	12	16	4.74	81.29
C	13	28	3.24	55.57
C	14	47	3.19	54.71
C	15	69	4.12	70.66
C	16	82	4.58	80.55
C	17	133	0.67	11.49
C	18	142	0.31	5.32
C	19	180	0.31	5.32

SUMMARY

1. The heterogeneous character of the potato starch paste in the United States Pharmacopœia for the assay of amylase activity under Pancreatin makes it very difficult to get uniform reaction mixtures. This condition may be greatly improved by homogenizing the starch paste.

2. The starch iodine end point of the official assay is unsatisfactory for the following reasons:

(*a*) One part of starch in a 2000 dilu-

tion cannot be differentiated from the standard iodine solution.

(*b*) The starch iodine reaction is very sensitive to light.

(*c*) The temperature of the iodine solution not only influences the color and intensity, but also the time involved in reaching the end point.

(*d*) The end point is not a true indicator of amylase activity, since it does not register the termination of this hydrolytic reaction.

3. Pancreatic amylase is relatively stable over long periods of time when properly stored. Samples of good appearance varying in age from sixteen months to eleven years had practically the same enzyme activity. Other samples of similar age but of poor appearance had a much lower activity.

4. If the official assay is retained, it is recommended that the following modifications of the official monograph of Pancreatin be made:

(*a*) The starch paste used should be homogenized. There is no good reason to insist on the use of potato starch.

(*b*) The wording of the end point "*no blue or violet color is produced*" be changed to *no color is produced which can be distinguished from that of the untreated iodine solution.*

(*c*) The official definition of amylase activity, wherein it states "*Pancreatin converts not less than 25 times its weight of starch into soluble carbohydrates,*" be modified by adding the phrase, "*in 5 minutes.*"

(*d*) It is further suggested that these modifications be observed in the official assay of Extract of Malt and that the definition of amylase activity for this preparation be modified to read, *Extract of Malt is capable of converting not less than five times its weight of starch into water soluble sugars in 30 minutes.*

5. The modifications suggested in 4 *c.* and *d.* should be adopted in order to avoid the misinterpretation which is commonly made because the time element is not conspicuously recorded.

6. The substitution of different starches or components of starch for the soluble starch by the method of Willstätter, Waldschmidt-Leitz, and Hesse (13) gave practically the same pancreatic amylolytic activity. This is an advantage over the official method which specifies the use of *potato starch.* Furthermore the use of activators, the control of *p*H, and the determination of the amount of maltose as the end product give this method added advantages over the official method.

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WHO MAKES IT?

The National Registry of Rare Chemicals, Armour Research Foundation, 33rd, Federal and Dearborn Streets, Chicago, Ill., seeks information on sources of supply for the following chemicals:

l-Ornithine
Sparassol
6-Methylsalicylic acid
Papaveraldine
Laudanosine
Codamine
Carnegine
Pellotine
d- and *l*-Arabitol
Trihydroxyglutaric acid
Phosphopyruvic acid
dl-Phenylglyceric acid
Pregnanediol
Isoandrosterone
Dehydroandrosterone
2-Aminobutyrolactone hydrobromide
5-Chloropentanone-2
Emetine
Dimethylhydroxylamine
d- and *dl*-Cysteine hydrochloride

A Study of the Tryptic Activity of Pancreatin U. S. P.*

By CHARLES W. BAUER† and ARTHUR K. WHITE‡

The official assay for pancreatic trypsin is not an assay for a single enzyme but for a mixture of proteolytic enzymes. Many samples labeled Pancreatin U. S. P. possess three times the minimum U. S. P. trypsin requirements. Triple-strength pancreatin is frequently no stronger than ordinary U. S. P. Pancreatin.

PANCREATIN is described in the United States Pharmacopœia as "a substance containing enzymes, principally pancreatic amylase, trypsin, and pancreatic lipase, obtained from the fresh pancreas of the hog .....". Without mentioning trypsin directly, but only by inference, the monograph further states that "pancreatin converts not less than 25 times its weight of casein into proteoses."

"Trypsin" is the name coined by Kühne (1) in 1867 for a proteolytic enzyme found in pancreatic juice. It means "a substance that cleaves." Kühne also used the name "Pancreatin" for the trypsin-containing precipitate that he obtained when he treated the pancreatic extract with alcohol.

The proteolytic effect of pancreatic juice was first recognized by Purkinje and Pappenheim (2) as early as 1836. A more comprehensive study than theirs of the effect of pancreatic juice on egg albumin was made by Corvisart (3) in 1856. However, it remained for Kühne (1) and his co-workers to recognize the fact that the proteolytic enzyme or enzymes in pancreatic juice carried the decomposition of egg albumin beyond the peptone stage. They found that leucine and tyrosine were among the products of decomposition when peptones were treated with either pancreatic juice or minced pancreatic extract.

Northrop and Kunitz (4) obtained a crystalline proteolytic enzyme from the pancreas of cattle in 1932. They retained the name "Trypsin" to designate "the most important proteinase" found in the pancreas.

The trypsin found in Pancreatin U. S. P. is the proteolytic enzymes, that convert a special preparation of casein into decomposition products that are not precipitated by a specially prepared mixture of acetic acid and alcohol. The official assay is based upon the formation of proteoses by this process; but since trypsin, as Kühne (1) has shown, actually converts proteoses into simpler compounds, it appears advisable to base the assay, not upon proteoses, as is done in the Pharmacopœia, but upon the simpler compounds beyond the proteose stage, the amino acids. These can be assayed by Sørenson's Formol Titration Method (6).

The assay methods for trypsin found in pharmaceutical literature are varied and difficult to interpret. For example, the United States Pharmacopœia states that pancreatin will digest 25 times its own weight of casein into proteoses. *New and Nonofficial Remedies* (1941) states that one part of Trypsin-Armour "digests at least 100 parts of casein according to the Fuld–Gross method." Trypsin–Fairchild, according to the same authority, when treated by the Fuld-Gross method will convert "200 times its weight of casein to the standard end-point." From these statements one may be led to believe that the latter preparation is 8 times as potent as Pancreatin U. S. P. even though the assertion is not directly made. When these preparations are assayed by identical methods, as by the Formol Titration Method, a true comparison can be made, and it will be found that the potencies of these preparations do not vary in the proportion implied.

The assay method of trypsin given in the United States Pharmacopœia under pancreatin is based upon the digestion of casein by proteolytic enzymes for a definite period of time, to the stage where the digestion mixture fails to give a precipitate when treated with a specially prepared mixture of acetic acid and alcohol. The acetic acid mixture

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‡ This paper is based on a thesis presented to the Graduate Council of the Massachusetts College of Pharmacy by Arthur K. White, in partial fulfillment of the requirements for the degree of Master of Science in Pharmacy.

first powdered and sifted through a number 100 mesh sieve before it was treated with the solvent. When this powder was used a good suspension was formed, which was used with consistently satisfactory results Pancreatin U. S. P. was assayed for casein digestive power with four different samples of casein by this modified method with nearly identical results. This indicates that the commercial samples of casein are similar in character to one another, and that they will give nearly homogeneous suspensions if the casein is first powdered and passed through a number 100 mesh sieve. All the experimental work, however, which followed, was done upon the same sample of casein.

Since it is impossible to put casein into solution by the U. S. P. method or by the Willson method, it appears advisable to modify the accepted procedure in order to bring about a more nearly homogeneous suspension of this substrate.

The Tryptic Activity of Various Samples of Commercial Pancreatin.—The discovery of pancreatic fibrosis and the renewed interest in pancreatic therapy for this condition, i.e., the inability to secrete trypsin, make it very important to know the tryptic activity of the available preparations of pancreatin or of the trypsin in order properly to estimate their medicinal value.

Commercial samples of pancreatin were assayed, in order to determine the exact amount of tryptic activity in them, by the modified Willson method instead of by the official assay for the minimum requirements indicated in the U. S. P. definition of pancreatin. If a sample is called "Triple Strength" Pancreatin, does it mean that it has three times the strength of the minimum requirements, or does it mean that it is three times as potent as ordinary Pancreatin U. S. P.? The U. S. P. test fails to distinguish between a sample which meets the minimum requirements and one which may be many times as potent.

TABLE IV.—COMMERICAL SAMPLES OF PANCREATIN ASSAYED BY THE MODIFIED WILLSON METHOD

Co.	Sample No.	Age in Mo.	Cc. of 0.1 *N* NaOH Used in Assay	Cc. of 0.1 *N* NaOH Used in Blank	Cc. of 0.1 *N* NaOH Actually Used
A	20	26	9.01	3.47	5.54
A	1A	24	9.55	2.97	6.58
A	11A	11	9.50	2.90	6.60
B	17	18	7.43	2.43	5.00
B	2A	67	7.03	2.28	4.75
B	3	67	7.18	2.57	4.61
B	3A	103	6.39	2.77	3.62
B	8A	67	6.98	2.72	4.26
C	10A	35	7.57	2.52	5.05
C	4A	53	7.47	2.47	5.00
C	4	89	7.77	2.57	5.20
C	12	23	7.60	2.68	4.92
C	3X[a]	Age unknown	10.36	3.28	7.08
D	7A	112	7.77	2.52	5.25
D	14	100	7.03	2.52	4.51
D	3Y[b]	Age unknown	10.58	3.37	7.21

[a] "Triple Strength" Pancreatin.
[b] "Triple Strength" Enteric Coated Capsule.

TABLE V.—SAMPLES OF TRYPSIN OBTAINED FROM VARIOUS SOURCES ASSAYED BY THE MODIFIED WILLSON METHOD

Sample No.	Cc. of 0.1 *N* NaOH Used in Assay	Cc. of 0.1 *N* NaOH Used in Blank	Cc. of 0.1 *N* NaOH Actually Used
1	9.06	3.14	5.02
1A	8.91	3.15	5.76
2	9.30	3.00	6.30
3[a]	10.25	3.46	6.79
3A[a]	10.30	3.59	6.71
4[b]	9.45	3.15	6.30

[a] "Triple Strength" Enteric Coated Tablets.
[b] Reference Standard Trypsin.

By assaying by the modified Willson method the samples of pancreatin obtained from different companies, it was found that most samples were two to three times as strong as the minimum requirements indicated on the label. Furthermore, in comparing a number of samples of Pancreatin U. S. P. (Company C) with a sample of "Triple Strength" Pancreatin from the same company, it was found that the U. S. P. samples were of approximately 70% the strength of the "Triple Strength" sample; whereas it would seem that they should be more nearly 33% as strong as the "Triple Strength" Pancreatin. However, a noticeable difference in the samples can be determined by the modified Willson method, whereas a previous experiment, when Pancreatin U. S. P. and "Triple Strength" Pancreatin were assayed by the U. S. P. method, merely showed that they both met the U. S. P. minimum requirements.

SUMMARY

1. The trypsin designated in the U. S. P. Pancreatin is a mixture of proteolytic enzymes.

2. The official assay is unsatisfactory, as no definite standard, with which different samples can be compared, is set up.

3. A modification of Willson's assay of pancreatin has been undertaken and has worked out successfully.

4. A number of commercial samples of pancreatin have been obtained and assayed by this method. A comparison of the strength of trypsin in various samples of pancreatin can be expressed in terms of the number of cc. of 0.1 *N* sodium hydroxide used to neutralize the amino acids produced

by digestion upon a specially prepared solution of casein.

5. A modification of both the U. S. P. and the Willson methods has been made in the preparation of the casein solution.

6. Samples of commercial trypsin were also assayed by the modified Willson method, and the results have been compared with U. S. Pharmacopœia Reference Trypsin.

7. It has been demonstrated by these assays that "Triple Strength" Pancreatin possesses three times the potency of the minimum requirements as stated in the official monograph; but, that it is misleading to imply that it posesses three times the potency of Pancreatin U. S. P.

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The Carboxypeptidase Activity of Pancreatin U. S. P.*

By CHARLES W. BAUER† and ALPHONSE M. MISKINIS‡

Pancreatin U. S. P. contains the enzyme carboxypeptidase. It is a relatively stable enzyme. Pumpkin seed globulin serves as an excellent substrate for its analysis. Its activity may be expressed in terms of cc. of 0.02 *N* sodium hydroxide required to neutralize the amino acids produced from a definite amount of pumpkin seed *alpha*-globulin.

UNTIL a few years ago it was thought that proteins were first digested to peptones by the pepsin in the stomach, and were then completely digested into absorbable units by the trypsin of the pancreas. It is now known that the enzyme trypsin, in the strict meaning of the term, does not complete the digestion of proteins into absorbable units. It has been found that the enzymes aminopeptidase and carboxypeptidase accomplish this result. Since carboxypeptidase is secreted by the pancreas, it is of interest to determine whether this enzyme is present in the official preparation of pancreatin.

The object of this investigation was to determine the presence and the relative amounts of carboxypeptidase in different samples of Pancreatin U. S. P.

In 1927 Waldschmidt-Leitz (1) and coworkers discovered the enzyme carboxypolypeptidase as the zymogen in the pancreas of certain of the higher animals. Two years later Waldschmidt-Leitz and Purr (2) shortened the name of carboxypolypeptidase to carboxypeptidase.

According to Bergmann (3) carboxypeptidase splits an end amino acid from its substrate, the enzyme attacking only those substrates containing a free carboxyl group next to the peptide linkage. The work of Bergmann and Fruton (4) indicates that the CO—NH—CH(R)—COOH groups are arranged in a counterclockwise order in which the alpha hydrogen atom of the CH(R) group is directed toward the enzyme. Anson (5) found carboxypeptidase to be the only known proteolytic enzyme that acts in the presence of formaldehyde.

One of the synthetic substrates employed for the measurement of carboxypeptidase activity is the expensive chloracetyl-*l*-tyrosine which was used by Waldschmidt-Leitz (2). Anson (7) found that a peptic digest

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of edestin was a satisfactory substrate for his investigations of carboxypeptidase.

Waldschmidt-Leitz (1) asserts that the zymogen found in the pancreas is activated by the enterokinase of the intestines. It has been suggested by Sumner and Somers (6) that the activation of the zymogen may be due to the action of trypsin.

MATERIALS AND METHODS

Substitute Substrate for Edestin.—Since chloracetyl-*l*-tyrosine was too expensive to be used in this investigation, the peptic digest of edestin was first employed as the substrate. The formalized solution of edestin was prepared according to Anson (7), using edestin (Eimer and Amend), decolorizing charcoal (Merck), granulated pepsin (Wilson Laboratories), and Filter-Cel (Johns-Manville). After several preliminary experiments with the edestin solution, the supply of edestin ran out, and no more could be obtained anywhere. The experiments performed with edestin clearly indicated the presence of carboxypeptidase in Pancreatin U. S. P. In order to continue this investigation it became necessary to find a satisfactory substrate to take the place of edestin.

In 1940 Vickery, Smith, and Nolan (8) reported the use of pumpkin seed globulin as a substitute for the globulin edestin obtained from hemp seed. These workers found the yield of globulin prepared from pumpkin seed by their method to be about 10%, by weight of the seed, a percentage somewhat higher than that of hemp seed. An added advantage of pumpkin seed globulin is that it is cheaper than edestin. Hence, pumpkin seed globulin was used as the substrate for the major portion of the experimental work reported in this paper.

Theory of Formol Titration and Modification.—In the determination of proteolytic enzymes other than carboxypeptidase by formol titration, the usual procedure for obtaining a standard consists of adding formaldehyde to the substrate solution to stop the enzymatic digestion, then of adding phenolphthalein and a sufficient amount of sodium hydroxide to bring the solution to a standard color. This preparation constitutes the standard or control. The increase in amount of alkali used over that of the control is a measure of the number of amide linkages split. This procedure is inadequate, according to Anson (7), and has to be slightly modified for the determination of carboxypeptidase, since the carboxypeptidase activity, unlike other proteolytic activities, is not stopped by the addition of formaldehyde. The blank is run on a substrate solution in the absence of the enzymatic preparation but in the presence of formaldehyde, phenolphthalein, and sodium hydroxide.

In the experimental investigation which follows, the blank was further modified by adding a boiled enzyme preparation which contained some amino acids not obtained from carboxypeptidase digestion. This modification eliminates the danger of including the amino acids, which are normally present in the crude pancreatin, from being considered to be the result of enzymatic action upon the substrate.

Preparation of Pancreatin Suspension.—A 5% pancreatin suspension was found by trial to be very satisfactory for this investigation. The suspension of pancreatin should be as homogeneous as possible in order that equal measurements may contain about equal amounts of pancreatin. All suspensions were made in distilled water about one half-hour before starting the assays.

Since heating might possibly have hydrolyzed some additional acids in the pancreatin, it was necessary to determine the acidity of both the boiled and the unboiled pancreatin suspensions. The effect of heat on the hydrolysis of pancreatic proteins had to be determined in order to have a correct control or blank. Phenolphthalein was used as the indicator, and the boiled suspension was first cooled to room temperature before assaying. The experimental results indicate that boiling has no effect upon the acidity of the preparation, as is shown by the fact that the amount of alkali was practically the same in each instance.

While the acidity of different samples of pancreatin may vary, the difference between the boiled and unboiled pancreatin in any one sample is about the same.

Preparation of Standard Formaldehyde Solution.—To 50 cc. of water add 15 cc. of formaldehyde 37%, 2 cc. of 0.5% phenolphthalein indicator solution, enough 5 *N* sodium hydroxide to make the solution just detectably pink, and enough distilled water to make the total volume measure 100 cc. This solution contains the same amount of formaldehyde as the formalized substrate solution.

Preparation of Pumpkin Seed Globulin.—To 150 Gm. of freshly ground pumpkin seed, in a previously warmed dry mortar, add sufficient 10% sodium chloride solution, warmed to 60–65°, to form a very soft paste. Triturate; add about one liter of sodium chloride solution and triturate for an additional five minutes. Strain through a number 20 sieve into a large evaporating dish and return the residue to the mortar for a second treatment. Use the remainder of the sodium chloride solution, two liters of which are prepared for the preparation, for the second extraction. Triturate again; then strain the contents into the large evaporating dish. Heat for one hour at 60–65° with constant stirring. Transfer the contents to a two-liter flask and tie a double fold of cheesecloth over the neck of the flask in the form of a small sack.

Invert the flask and contents into a stepfolded filter paper that has been washed with warm 10% solution of sodium chloride. It is desirable to use a water-jacketed funnel, maintained at 60–65°, for the filtration process, for, if the funnel and paper become cold, the globulin will precipitate and will clog the pores of the filter paper.

Dilute the filtrate with distilled water to a 2% concentration of sodium chloride and set it aside at room temperature for four to five hours; then place it in the refrigerator at 5° for twelve hours. Then siphon off the supernatant liquid. Filter the precipitate through a large Jena filter or a glass sintered filter, and wash the precipitate twice with a small amount of distilled water. Dry the precipitate over a steam-heated radiator or in a warm place, powder it finely, and pass it through a bolting cloth. The extracted globulin is light yellow-brown in color and behaves very similarly to edestin, as a control experiment showed. The yield by the foregoing method is about 6–7%.

The pumpkin seed globulin served as a satisfactory substrate for the determination of carboxypeptidase activity in Pancreatin U. S. P. Anson's (7) procedure was used to prepare the formalized substrate solution, but pumpkin seed globulin was substituted for the edestin.

Preparation of Digestion and Control Flasks and the Method of Assay

The Preparation of the Standard Color for the Blank.—To flask 1S, the standard color flask, add 10 cc. of 5% boiled pancreatin suspension previously cooled to room temperature, one drop of phenolphthalein indicator, and titrate to a pink color with 0.02 *N* sodium hydroxide in order to neutralize the amino acids present in the pancreatin suspension. Then add 5 cc. of neutralized standard formaldehyde solution. The pink color disappears on the addition of the formaldehyde solution because of the release of the carboxyl groups of the amino acids present as zwitter ions in the neutralized pancreatin. Make the suspension pink again with 0.02 *N* sodium hydroxide. Stopper the flask, and let this preparation represent the color standard for flasks 2a and 2b.

Blank Determination of Flasks 2a and 2b.—Add 10 cc. of 5% cooled, boiled pancreatin suspension, and one drop of phenolphthalein indicator to flasks 2a and 2b, which are to be employed as blanks for the digestion flasks 4a and 4b. Titrate the mixtures with 0.02 *N* sodium hydroxide to the same pink color as that of the standard color flask, 1S; then add 5 cc. of formalized substrate solution. The pink color disappears after this addition, but returns on the addition of more alkali. After the desired pink color is obtained, stopper the flasks and place them in a constant temperature bath for thirty minutes at 37°. The standard color flask, 1S is also placed in the constant temperature bath at the same time. The amounts of 0.02 *N* sodium hydroxide used need not be recorded, for the alkali is used only to neutralize the acids in the pancreatin suspension as well as any amino acids that may be liberated when the formaldehyde solution is added. None of these acids is a product of the digestion with the enzyme preparation.

At the end of thirty minutes—the period allowed for digestion in flasks 4a and 4b—remove the flasks from the temperature bath. Titrate the contents of the flasks 2a and 2b with 0.02 *N* sodium hydroxide until the color of each matches that of the standard flask. Record the number of cc. of 0.02 *N* sodium hydroxide required to produce the desired color in flasks 2a and 2b. This is the amount, in terms of cc. of 0.02 *N* sodium hydroxide, necessary to neutralize the acids that may be produced during the thirty-minute digestion period. Theoretically there should be no amino acids produced, since the pancreatin suspension was boiled for seventy-five seconds to destroy all enzymatic activity. Flasks 2a and 2b are employed as blanks for the digestion flasks 4a and 4b, which contain the unboiled, enzymatically active, pancreatin suspension.

The Preparation of the Standard Color for the Digestion Flasks.—To flask 3S, which is to be the standard color flask for flasks 4a and 4b, in which digestion occurs, add 10 cc. of unboiled 5 per cent pancreatin suspension, one drop of phenolphthalein indicator, and titrate to a pink color with 0.02 *N* sodium hydroxide. Add 5 cc. of neutral standard formaldehyde solution and again add 0.02 *N* sodium hydroxide until the standard color is obtained. Stopper the flask, and use it as the standard color flask for 4a and 4b.

The Preparation of the Flasks 4a and 4b.—Add 10 cc. of unboiled 5% pancreatin suspension to flasks 4a and 4b; then add one drop of phenolphthalein indicator and titrate to the standard color. Then add 5 cc. of neutral formalized substrate solution and immediately titrate to the standard color; stopper, and place the three flasks in the constant temperature bath at 37° for thirty minutes. The amount of sodium hydroxide is not recorded. However, after the removal of the flasks from the temperature bath, the number of cc. of 0.02 *N* sodium hydroxide required is an indication of the degree of carboxypeptidase activity in the pancreatin suspension.

At the end of thirty minutes remove the flasks from the thermostatic bath. If the color of the standard color flask, 3S, be markedly faded or be of such a weak shade that it is difficult to compare or reproduce, introduce a known amount of 0.02 *N* sodium hydroxide until the desired color is obtained. Titrate the other flasks, 4a and 4b, with 0.02 *N* sodium hydroxide until their color matches that of the standard (3S). Subtract the number of cc. of 0.02 *N* sodium hydroxide used to produce the desired color in the standard color flasks, 3S, from the total number of cc. used in each of the two digestion flasks 4a and 4b.

Interpretation of the Assay Described.—Boiling the pancreatin suspension for seventy-five seconds destroys the enzyme present in the blank flasks 2a and 2b. Therefore, if the average number of cc. of 0.02 *N* sodium hydroxide used in flasks 2a and 2b equals the average number used in 4a and 4b, which contain the unboiled pancreatin suspension, the activity shown would not be caused by the enzyme, but by something else. However, if the average amount of 0.02 *N* sodium hydroxide used in 2a

and 2b be less than the average amount used in 4a and 4b, it may be assumed that part or all of the activity was caused by the carboxypeptidase, because carboxypeptidase is the only known proteolytic enzyme that will act in the presence of formaldehyde.

Experimental Data.—A sample of Pancreatin U. S. P. was obtained from one of the larger pharmaceutical houses The determination of carboxypeptidase was determined by the previously described method of assay. The average requirement of the control flasks was 0.7 cc. of 0.02 *N* sodium hydroxide while the average requirement of the digestion flasks was 5.9 cc. The control average, 0.7 cc., subtracted from the digestion flask average, 5.9 cc., gives 5 2 cc of 0 02 *N* sodium hydroxide used. This is the actual carboxypeptidase activity measured in terms of cc. of 0.02 *N* sodium hydroxide.

TABLE I.—THE AMOUNT OF CARBOXYPEPTIDASE IN SAMPLES OF PANCREATIN OBTAINED FROM COMPANIES A, B, C, AND D EXPRESSED IN TERMS OF CC. OF 0.02 *N* SODIUM HYDROXIDE REQUIRED TO NEUTRALIZE THE ACIDS LIBERATED BY THE ENZYME

Co.	Sample No.	Age in Mo.	Av. No. of Cc of 0.02 *N* NaOH for Blanks	Av. No. of Cc. of 0.02 *N* NaOH for Digestion Flasks	Av. Carboxypeptidase Activity in Terms of Cc. of 0.02 *N* NaOH
A	1A	24	0.99	5.275	4.285
A	11A	11	1.15	5.190	4.040
B	4A	53	0.775	5.300	4.525
B	10A	35	0.675	5.275	4.600
B	12	23	0.500	5.400	4.900
B	12A	21	0.600	5.120	4.520
C	7	66	0.685	5.335	4.650
C	7A	112	0.900	5.350	4.450
C	14	100	0.400	5.700	5.300
C	15	46	1.115	6.100	4.985
D	3	67	0.700	5.650	4.950
D	2A	67	0 700	5.900	5.200
D	3A	103	0.635	6.100	5.465
D	17	18	0.800	6.150	5.350
D	5	73	0.410	5.950	5.540
D	8A	67	0.475	6.010	5.535
D	6	163	0.690	5.650	4.960
D	6A	85	1.060	5.500	4.440

The most recently manufactured sample of pancreatin assayed was eleven months old, the oldest sample was 163 months old, while the average age of the samples was about 53 months. The average carboxypeptidase activity of 10 cc. of a 5% suspension of pancreatin on 5 cc. of a pumpkin seed globulin substrate made according to the Anson method, expressed in terms of 0.02 *N* sodium hydroxide solution is 4.71 cc.

CONCLUSION

The experimental data definitely indicate the presence of carboxypeptidase in Pancreatin U. S. P. Since Pancreatin U. S. P. contains trypsin, a carboxypeptidase activator, it was impossible to determine the amount of enzyme in the form of its zymogen. The assay is based entirely upon active carboxypeptidase. Since all experimental work was carried out on an impure enzymatic preparation, it was impossible to express the amount of enzyme in Anson units (7), which are based on a crystalline carboxypeptidase. However, the amount of carboxypeptidase may be expressed in terms of the cc. of 0.02 *N* sodium hydroxide required to neutralize the amino acids liberated by carboxypeptidase from a definite amount of substrate. The amount of carboxypeptidase in samples of Pancreatin U. S. P. seems not to be affected to any significant extent by the age of the samples.

SUMMARY

1. Pancreatin U. S. P. contains carboxypeptidase.

2. Pancreatin suspensions may be boiled to destroy the enzyme without affecting the degree of acidity.

3. Pumpkin seed globulin is a satisfactory substrate for the determination of carboxypeptidase.

4. Boiled Pancreatin must be used in the control in order to avoid a misinterpretation of the carboxypeptidase activity of the unboiled pancreatin on the pumpkin seed globulin.

5. The activity of carboxypeptidase may be expressed in terms of cc. of 0.02 *N* NaOH required to neutralize the amino acids produced from a definite amount of substrate.

6. The amount of carboxypeptidase in samples of Pancreatin U. S. P. was practically the same for all samples, which were obtained from different sources, and which were stored for different periods of time. This uniformity seems to indicate that carboxypeptidase is a fairly stable enzyme.

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Chlorosulfonic Acid in the Synthesis of DDT and Its *p*-Halogen Analogues*

By W. T. SUMERFORD†

The chlorosulfonic acid method of preparing DDT is outlined, its general application discussed, and the compounds which were prepared are described.

SINCE the discovery of the insecticidal activity of DDT [1-trichloro-2,2-*bis*(*p*-chlorophenyl)ethane] by Müller (1) in 1939, the chemistry of this and related compounds has been developed in this country and elsewhere at a very rapid rate. Among the first analogues of DDT to suggest themselves as potentially superior substitutes were its *p*-halogen analogues: (*a*) 1-trichloro-2,2-*bis*(*p*-fluorophenyl)ethane (DFDT), (*b*) 1-trichloro-2,2-*bis*(*p*-bromophenyl)ethane (DBrDT), and (*c*) 1-trichloro-2,2-*bis*-(*p*-iodophenyl)ethane (DIDT).

Baeyer was the first to condense two moles of benzene with one mole of chloral to give 1-trichloro-2,2-*bis*(phenyl)ethane (2) herein referred to as (DPE).

Both DDT and its *p*-bromine analogue (DBrDT) were prepared and described by Zeidler in the first report on compounds of the DDT type (3).

The *p*-fluorine analogue of DDT (DFDT) was introduced in Germany during World War II under the names of "Fluorogesarol" (GIX) (4). It is said to be seven times as effective against flies and to cost ten times as much as DDT (5). DFDT was prepared by condensing two moles of fluorobenzene and one mole of chloral using sulfuric acid (4), or by condensing two moles of fluorobenzene and one mole chloral acetal in the presence of chlorosulfonic acid (6). It has been described as a liquid, b. p. 160–180° at 8 mm. (7) and as a solid, m. p. 31° (8). No analyses for this compound were found.

It has been shown that iodobenzene readily condenses with chloral with the formation of 1-trichloro-2,2-*bis*(*p*-iodophenyl) ethane (DIDT), colorless prisms, m. p. 172° (9).

All of the above condensations, unless otherwise noted, were brought about by the use of concentrated sulfuric acid. The order of mixing the reactants and the acid, however, was not the same in each case.

EXPERIMENTAL

The use of chlorosulfonic acid in condensing two moles of chlorobenzene and one mole of chloral to yield DDT, first reported from this Laboratory (10), has been extended to the synthesis of 1-trichloro-2,2-*bis*(phenyl)ethane and its *p,p'*-dihalogen derivatives.

The procedure used to prepare and purify these derivatives is essentially the same as that previously described (10). One mole of chloral is mixed with 2 moles of benzene or the appropriate halogen derivative. This solution is cooled to 5° and to it is added 1.8 moles of chlorosulfonic acid in divided portions and under constant stirring. After stirring for three hours, the reaction mixture is poured into chipped ice and the crude product is collected on a Buchner funnel. It is boiled with ten times its weight of water and again collected after cooling.

The pertinent data for the compounds prepared by this method are given in Table I.

TABLE I

Compound	Recrystallizing Solvent	Yield, %	M. P., °C
DPE	$MeOH—H_2O$[b]	60	62
DFDT[a]	$EtOH—H_2O$[b]	68	40
DDT	$Et_2O—MeOH$ (1–3)[c]	70	106
DBrDT	$Et_2O—EtOH$ (2–1)	75	139
DIDT	$Et_2O—EtOH$ (1–1)	80	172

[a] This compound was solidified prior to recrystallization by placing it in a chest with dry ice. Theory: C% 52.20 H% 2.80. Found: C, 51.72. H, 2.80.
[b] Dissolve in appropriate amounts of the respective alcohols, and add water to incipient precipitation.
[c] This compound may be purified also by washing it twice with an equal weight of methanol.

Biological Activity.—Determinations have been made of the relative toxicity of these compounds

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toward mosquito larvae, mosquito pupae, and two species of fish. The unsubstituted compound, DPE, was comparatively nontoxic. The median lethal doses of the *p,p'*-dihalogen derivatives were approximately 0.1 p. p. m. for goldfish, 0.01 p. p. m. for Gambusia, and 0.001 p. p. m. for larvae. The pupae were more resistant than the larvae or the Gambusia

A detailed report on these tests will be published elsewhere.

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Book Reviews

Advancing Fronts in Chemistry. Volume II, Chemotherapy. Edited by WENDELL H. POWERS. Reinhold Publishing Corporation, 330 West 42nd St., New York, 1946. 156 pages. 14.5 x 23.0 cm. Price, $3.50.

In 1945 the Wayne University sponsored a course of academic studies entitled "Frontiers in Chemistry." It was certainly fitting that chemotherapy, a science which has given so much to the health of mankind, should fit into such a program. This book is a compilation of all of the lectures presented in the chemotherapeutic field with the exception of Dr. H. E. Carter's lecture on antibiotics. The following lectures were included:

"Chemotherapy in Experimental Tuberculosis," by William H. Feldman;
"Synthetic Antispasmodics," by Frederick F. Blicke;
"Chemistry of the Sulfa Drugs," by E. H. Northey;
"The Antimalarial Problem," by Harry S. Mosher;
"Organometallic Compounds as Chemotherapeutic Agents," by C. Kenneth Banks;
"Past Developments and Present Needs in the Chemotherapy of Parasitic Diseases," by Willard H. Wright.

In all cases, the emphasis has been placed on recent developments and no attempt was made to completely survey the literature in these specific fields. Enough historical material was presented to provide a satisfactory setting. Many of the authors were particularly stimulating by indicating probable future trends and future needs.

The inclusion of photographs of the authors and brief biographies will be a delight to many since they help create the illustion that the reader attended this auspicious lecture series.—MELVIN W. GREEN.

Chemistry of Food and Nutrition, Edition 7, by HENRY C. SHERMAN. The Macmillan Company, New York, 1946. viii + 675 pages. 13.5 x 21 cm. Price, $3.75.

Nutrition as a chemical science and Professor Sherman's excellent text cannot be separated without leaving a tremendous void. It is difficult to realize that the first *Chemistry of Food and Nutrition* became available in 1911. Many spectacular and dramatic events have taken place in the intervening years. Professor Sherman has not only been most faithful in recording these events, he has actively participated in most of them.

The seventh edition of this book, like the others represents a thorough revision where swift moving events demanded it. Because the author has taken pains to make clear the certainty degree of his "facts," the undergraduate is not at a loss to know the "truth."

The documentation is selective and exceptionally good.—MELVIN W. GREEN.

Qualitative Analyses by Spot Tests, by FRITZ FEIGL. Third English Edition, 1946. Elsevier Publishing Company, New York. xvi + 574 pages. 14.5 x 23 cm. Price, $8.00.

The third English edition of this standard work on spot testing is much like previous editions in form. It has been completely revised, however. Not only have new tests been added but the tests previously given have been critically evaluated. All manipulations previously described separately in *Laboratory Manual of Spot Tests* (Academic Press, 1943) have been included. Altogether the text contains 350 detailed and about 100 orienting tests in addition to about 175 suggested procedures. This edition was translated by Professor Ralph E. Oesper.—MELVIN W. GREEN.

Scientific Edition

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Quantitative Assay of the Intravenous Toxicity of Streptomycin in Mice*

By WALTHER H. OTT

A method has been devised for the quantitative assay of the intravenous lethal toxicity of commercial streptomycin in mice whereby the LD_{50} is determined from the observed mortality by direct reference to tabulated values. Using certain specified doses and 5 mice per dose, two designs for the assay are presented: a two-dose method with a standard error of approximately ±8% of the LD_{50}, and a single-dose method with a standard error of ±10%.

THE NEED for a quantitative assay of the intravenous toxicity of commercial streptomycin in mice became apparent with the observation (1) that the intravenous toxicity in mice was unrelated to the potency of the drug and presumably was due to some substance, or substances, other than pure streptomycin. Although a safety test has been described by the Food and Drug Administration (2), this test shows only that the toxicity of the tested sample exceeds, or falls below, a certain limit approximately equivalent to an LD_0 of 1000 micrograms streptomycin base per 20-Gm. mouse. Therefore attention was directed toward development of a quantitative assay of reasonable accuracy using the technical specifications set forth by the F.D.A. for the safety test.

* Received March 7, 1947, from the Merck Institute for Therapeutic Research, Rahway, N. J.

MATERIALS AND METHODS

The mice used in these assays were of a uniform strain (CFI) obtained at weekly intervals from the Carworth Farms. The animals had continuous access to water and a nutritionally complete laboratory diet, and were housed in metal cages in quarters maintained at 75° F. Mice weighing from 19 to 24 Gm., inclusive, were used in all tests. The intravenous injection was made into a tail vein using a 1-ml. syringe with a 26 or 27 gauge needle. The injection time was kept as near as possible to five seconds with the aid of a metronome. All mice were observed continuously for thirty minutes, and the survivors were observed daily for two days. No mouse was ever used more than once.

In view of the fact that the experimental error of the median lethal dose, or LD_{50}, is less than that of any other lethal dose, especially the LD_0 (3), the LD_{50} was selected as the measure of toxicity. Biometrical methods described by Bliss, *et al.* (3, 4) were employed in analyzing and evaluating the data.

Using the above procedures, several experiments were conducted to study some of the factors influencing the test (Tables I and II). Five mice were used

on each dose in all but one of the experiments, in which 10 mice were used per dose. In each assay different doses were given until several were found that caused mortalities in the range between 0% and 100%. Within each comparison of relative toxicity (Table I) the various doses were prepared directly from the same stock solution of streptomycin. The factors other than the one being studied were kept constant from dose to dose within each assay. In addition to the factors listed, these included date of assay, age of mice, and individual injector.

Two strains of mice, CFW and CFl, were available, and they were found to differ significantly in resistance to the toxic properties of streptomycin. No explanation was sought for this difference. The CFl strain, being more readily available in large numbers, was adopted for all streptomycin toxicity assays in this laboratory.

Since mice differing by as much as 25% in body weight (usable range 19 to 24 Gm., inclusive) are employed in the assay, the relation between dose and body weight was investigated. When the dose of the drug was an amount calculated to be directly proportional to the body weight the toxicity of streptomycin was the same for the heaviest mice as for the lightest mice within the usable weight range. On the other hand, when the dose was kept constant regardless of body weight, an apparently lower toxicity value was obtained with the heavier mice. Therefore, in the conduct of the quantitative assay, the dose for each mouse was made proportional to the body weight by adjusting the volume of the prepared dose on the basis of 0.50 ml. per 20 Gm.

TABLE I.—INFLUENCE OF FOUR FACTORS ON THE ASSAY OF THE INTRAVENOUS TOXICITY OF STREPTOMYCIN IN MICE

No. Mice Used Per Dose	Total	Assay Series		Mean Relative Toxicity[a] of Streptomycin (with Standard Errors)
5	195	Injection time, sec.:	5	100%
			10	102 ± 7.4%
			20	94 ± 7.1%
			30	83 ± 5.2%
			60	68 ± 4.3%
5	55	Sex:	Males	100%
			Females	93 ± 6.0%
5	125	Strain:	CFl	100%
			CFW	126 ± 5.4%
10	180	Injection dose:	per mouse, 19–20 Gm.	100%
			per mouse, 23–24 Gm.	87 ± 3.5%
			per 20 Gm., 23–24 Gm.	101 ± 4.1%

[a] The toxicity in one group within each series was arbitrarily assigned a value of 100%, and the toxicities in the other groups were expressed as a percentage of that value.

TABLE II.—INTRAVENOUS TOXICITIES OF STREPTOMYCIN OF DIFFERENT ORIGIN

Lot	No. Mice Used	LD_{50} Micrograms Base/20 Gm.	Individual Slope	Agreement between Observations and Fitted Curve[a] χ^2	Probability
A	30	1440	13.1	3.5	0.45
B	30	1570	22.0	0.3	0.99
C	30	1130	19.2	2.7	0.60
D	30	2380	15.1	4.6	0.20

[a] Agreement is significant in each instance.

RESULTS

In making the transition from the slow rate of injection formerly used (1) to the rapid rate of 0.5 ml. in five seconds as adopted by the F.D.A. (2), it was found that the observed toxicity gradually increased as the injection time was shortened to about ten seconds (Table I). No further change in relative toxicity was apparent when the time was reduced to less than ten seconds.

The two sexes differed very slightly in sensitivity to the toxic factor, the females appearing to be more resistant than the males. Although this difference is not statistically significant and no difficulty has been encountered due to it both sexes have never been used on the same sample.

Four lots of streptomycin of different origins were assayed using approximately 10% increments in dose (Table II). Six doses, at least four of which gave mortalities between 0% and 100%, were used on each lot. Even though the preparations varied considerably in relative toxicity, the slopes of the four dosage-mortality curves did not differ significantly from parallelism ($\chi^2 = 1.5$, $df = 3$, $P = 0.66$). This observation is considered an indication that the toxic factors in the different materials produced qualitatively similar responses in the mice.

Although 5 mice seem to be a small number to use on each dose, this number has proved sufficient, presumably due in part to a high degree of uniformity among the mice. This was indicated by the fact that the observed mortalities for any of the four lots of streptomycin did not differ significantly from the composite curve (χ^2, Table II).

Data from the above assays and 10 routine assays selected at random were combined to obtain a satisfactory estimate of the slope of the curve relating logarithm of the streptomycin dose to per cent mortality in probits. As shown in Table III, the pooled slope for this entire series was calculated as 14.8. The individual assay curves were parallel within experimental error ($\chi^2 = 31$, $df = 45$), and consequently the slope of 14.8 has been used in the calculations in this paper and for the streptomycin toxicity assays conducted in this laboratory.

THE QUANTITATIVE ASSAY

Using the preceding information, the F.D.A. safety test was modified so as to systematize and standardize the procedure irrespective of the toxicity of the individual sample. The objective of the quantitative assay was the determination of the LD_{50} in a single assay with reasonable accuracy and without expenditure of unreasonable effort.

For these purposes certain selected doses were injected into mice, using 5 mice per dose, until two adjacent doses in the series were found such that two or fewer mice died at the lower dose and three or more mice died at the higher dose. The doses designated for use in this quantitative assay were spaced at approximately 20% increments and were as follows: 700, 850, 1000, 1200, 1450, 1750, 2100, 2500, and 3000 micrograms streptomycin base per 0.5 ml. (corresponding to values equally spaced on a logarithmic scale before rounding). No other doses were used routinely. The concentrations were given per 0.5 ml. because each dose was injected on the basis of 0.5 ml. of the prepared solution per 20 Gm. mouse. The selected doses were prepared as needed from a stock solution of the sample containing 3000 micrograms streptomycin base per 0.5 ml. Distilled water was used for all solutions.

Following injection of a lethal dose, death invariably occurred within thirty minutes, and usually within five minutes. In fact, among more than

TABLE III.—SUMMARY OF DATA FOR CALCULATION OF THE SLOPE OF THE DOSAGE-MORTALITY CURVE FOR TOXICITY OF COMMERCIAL STREPTOMYCIN IN MICE

Assay Series	No. of Curves	No. of Mice	[wx²]	[wxy]	Slope
Injection time	15	195	0.3212	4.8153	15.0
Sex	4	55	0.0916	1.2442	13.6
Strain (CFI)	4	65	0.0703	0.9311	13.2
Origin	4	120	0.1291	2.2252	17.2
Injection dose	9	180	0.1477	2.2023	14.9
Routines	10	150	0.2307	3.2573	14.1
All	46	765	0.9906	14.6754	14.8

TABLE IV.—QUANTITATIVE TWO-DOSE ASSAY OF STREPTOMYCIN TOXICITY IN MICE[a]

Dose pair		No. of mice dead out of 5 mice injected per dose				
		3	3 4	3 4 5	4 5	5
High	Low	0	1 or 0	2 or 1 or 0	2 or 1	2
850	700	850	810	770	740	700
1000	850	1020	970	920	880	840
1200	1000	1210	1150	1100	1050	990
1450	1200	1450	1380	1320	1260	1200
1750	1450	1760	1670	1590	1520	1440
2100	1750	2110	2010	1920	1830	1740
2500	2100	2530	2400	2290	2190	2080
3000	2500	3020	2870	2740	2610	2480

[a] LD_{50}'s in the body of the table correspond to the 9 possible mortality combinations in which 0, 1, or 2 mice died at the lower dose and 3, 4, or 5 mice died at the higher dose in each of the 8 dose pairs. An individual assay is completed when any of these mortality combinations are obtained with any one of the 8 dose pairs.

All values are micrograms base per 20 Gm. mouse.

Example: The 2100-microgram dose is injected, killing 3 out of 5 mice. The next lower dose must then be given, which is 1750 micrograms. This dose kills 1 out of 5 mice. The mortality combination is 3 at 2100 and 1 at 1750, $\binom{3}{1}$, which is found at the heading for the second mortality column. Opposite the 2100-1750 dose pair is found 2010, the LD_{50} corresponding to these results.

The inherent precision of this assay is relatively high ($1/b = 0.068$) compared to other all-or-none assays (5). For the 10 routine assays (Table III) 2 to 4 doses and an average of 15 mice were used per sample. The average standard error of the LD_{50} was calculated as ±6.3% of the LD_{50}, a value indicative of satisfactory reproducibility for this assay.

In order to insure that known and unknown factors would not interfere beyond the limits of experimental error with the day to day accuracy of routine assay results, a sample of commercial streptomycin was assayed daily as a working standard. Statistical quality control (6, 7) was maintained on the LD_{50} of this standard as a measure of the resistance of the different groups of mice used from day to day. Confidence limits were established at the 95% probability values (7), and samples were considered to have been assayed satisfactorily when the LD_{50} of the standard fell within these limits.

10,000 mice surviving this initial observation period, only 10 deaths in the following two days (all from one sample) were observed. No deaths occurred among approximately 2500 surviving mice which were observed for ten days after the injection. Accordingly, the mortality occurring in the initial observation period can be considered the final value for each dose, and the assay can be conducted from dose to dose without appreciable delay until the requirements for the design of the assay have been met.

The LD_{50} may then be read from Table IV as the value corresponding to the particular mortalities and pair of doses in each assay. This table was prepared for the 9 possible mortality combinations which can be obtained for each of the 8 dose pairs fulfilling the requirements set forth above for the quantitative assay. The LD_{50}'s in the table were computed for the corresponding pair of doses and mortalities using the slope of 14.8 and the methods described by Bliss (3).

Since the high precision of this assay is not required for many types of routine samples, the assay was simplified for such samples by designing a one-dose procedure. This was accomplished by omitting alternate levels in the series of doses employed in the two-dose assay, and by requriing that one of these doses be found which kills 1, 2, 3, or 4 out of 5 mice or that two adjacent doses in this particular series of doses (Table V) be found such that no mice die at the lower dose and all mice die at the higher dose.

TABLE V.—QUANTITATIVE ONE-DOSE ASSAY OF STREPTOMYCIN TOXICITY IN MICE[a]

Dose	No of Mice Dead Out of 5 Mice Injected				
	1	2	3	4	0^b——5^b
700	800	730	670	610	x (580−)
					840
1000	1150	1050	960	880	x x
					1200
1450	1650	1500	1400	1250	x x
					1750
2100	2400	2200	2000	1850	x x
					2550
3000	3400	3100	2900	2650	(3600+) x

[a] LD_{50}'s in the body of the table correspond to 4 mortalities possible with each dose and to one mortality combination possible with a pair of adjacent doses

All values are micrograms base per 20 Gm mouse.

Example: The 1450-microgram dose is injected, killing no mice A higher dose must be given. The 2100 dose is chosen which kills 3 out of 5 mice, thus completing the assay Opposite the 2100 dose in the column for 3 dead is found 2000, the LD_{50} corresponding to this result. If all 5 mice had died at the 2100 dose, the LD_{50} would be found in the 0——5 column as 1750.

[b] None dead at one dose and all dead at next higher dose

The occurrence of any one of these 5 possible mortalities completes this one-dose assay, and the corresponding LD_{50} is found by reference to Table V. In other respects this assay is performed in the same manner as the two-dose assay. Only doses selected from the 5 doses listed in Table V are used in this assay. The LD_{50}'s in this table were computed by means of the equation, $X = x + \frac{(5 - y)}{14.8}$, where X is the log LD_{50} corresponding to the per cent mortality in probits, y, and to the log dose, x (3).

DISCUSSION

With the assay using two doses and 5 mice per dose the average standard error of the LD_{50} was calculated as approximately ±8%. However, by running a duplicate assay on a sample the standard error of the average LD_{50} is reduced to approximately ±6%. In actual practice a standard error of ±4.2% of the average LD_{50} was found in duplicate assays conducted in the above manner on 50 samples during a period of almost three months. In this group of assays, duplicate values were obtained on 7 samples, and none of the differences between LD_{50}'s in duplicate assays on the remaining samples exceeded 18.8% of the average LD_{50} in each instance. Therefore, the actual reproducibility of the assay appears to be slightly better than that expected from the theoretical standard error of ±6%.

The standard error of the LD_{50} in the one-dose assay was calculated as approximately ±10%. Although a large number of samples have been assayed using the one-dose method, relatively few of the samples were reassayed because the agreement between duplicate assays has been very satisfactory. Among 10 samples reassayed in a four-month period, duplicate values were obtained on three samples, while a difference of 33% between LD_{50}'s in duplicate assays was found on one and differences ranging from 4% to 16% on the remaining six samples. This reproducibility is somewhat better than expected, as was also noted with the two-dose assay.

A minimum of 10 mice is needed for a single two-dose assay (5 mice per dose) and 20 mice for a duplicate two-dose assay. On the other hand, the minimum for the one-dose assay is 5 mice. However, it can be seen that not more than 15 mice (3 doses) need ever be used in the one-dose assay provided the 1450 microgram dose is the first dose administered. In actual routine use, an average of 7 mice has been used per sample with the one-dose method, and an average of 11 mice has been used per sample with the two-dose method. These results were accomplished with samples of relatively uniform, but unknown, toxicity ranging from 700 to 3000 micrograms streptomycin base per 20 Gm. mouse.

It is to be noted that quantitative results were obtained by the above assay methods on samples of unknown toxicity with the average expenditure of not more than twice the time and effort required for the purely qualitative safety test, namely 7 to 11 mice per sample for the quantitative assays as compared to 5 mice per sample for the safety test. The advantages of having available the LD_{50} of every sample have far outweighed the disadvantages of the extra work involved. Furthermore, the procedure for the quantitative assay has been designed so that the assay is as easy to conduct and evaluate as the safety test itself.

The LD_{50}'s in the tables for the quantitative assays (Tables IV and V) were computed for a dosage-mortality curve having a slope of 14.8, and are valid particularly for the assay of intravenous toxicity of commercial streptomycin in mice. As a matter of fact, the above tables could be used in the conduct and evaluation of assays of the toxicity of any drug exhibiting a slope of approximately 14.8 for the dosage-mortality curve. Since the slope of the dosage-mortality curve may vary from laboratory to laboratory, it is recommended that each laboratory determine the slope of the curve, and if it differs significantly from 14.8, the principles applied above be used to design similar tables for use under their particular conditions.

SUMMARY

1. An assay method of relatively high precision has been developed for the quantitative determination of the intravenous toxicity of commercial streptomycin in mice using certain specified doses and 5 mice per dose, and evaluating the LD_{50} from the resulting mortalities by direct reference to tabulated values.

2. Two designs for the assay are described: a two-dose assay with a standard error of approximately $\pm 8\%$ and a one-dose assay with a standard error of about $\pm 10\%$.

3. In routine assays using the two-dose assay, an average of 11 mice has been used per sample. Using the one-dose assay the average has been 7 mice per sample.

4. The slope of the dosage-mortality curve for this assay was estimated at 14.8.

5. The observed toxicity of streptomycin increased as the length of the injection period was shortened from one minute to ten seconds. No difference was found between the ten-second and five-second rates.

6. Females were not significantly different from males in resistance to the toxicity of streptomycin.

7. A significant difference in resistance was observed between the two strains of mice that were investigated.

8. The observed toxicity of streptomycin was relatively constant when doses of the drug were given in proportion to body weight in the range from 19 to 24 Gm.

9. No qualitative differences in toxicity were detected among four lots of streptomycin of different origins.

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Trimethyl Cetyl Ammonium Pentachlorphenate (TCAP) and Fatty Acids as Antifungal Agents*

By E. J. FOLEY and S. W. LEE

The antifungal activity of an experimental ointment and two solutions containing trimethyl cetyl ammonium pentachlorphenate was determined and compared with results obtained by similar methods using preparations containing fatty acids or their salts.

THE STUDIES of many authors (1–8, 15, 16, 24, 25) have established that fatty acids have marked antifungal properties, and therapeutic preparations of considerable merit containing fatty acids or fatty acid salts have recently been used for the treatment of dermatophytoses (9–12, 14) and tinea of the scalp (13).

In previous publications (15, 16) we have shown that trimethyl cetyl ammonium pentachlorphenate (TCAP) has marked fungicidal properties and that free fatty acids are far more effective *in vitro* against pathogenic fungi than are the salts of these acids. Of the higher fatty acids studied (heptylic, caprylic, pelargonic, capric, and undecylenic) the last appeared to be the most powerful antifungal agent. Appropriate experiments clearly indicated that by the use of vehicles which solubilize undecylenic acid or TCAP, their activity was greatly enhanced.

In order to obtain superior fungicidal action, it was advantageous to use the 2 agents, TCAP and undecylenic acid, in combination (24), and proceeding from the above facts, experiments were made to determine the best means of providing optimum conditions for fungicidal effect. *In vitro* tests were devised to study the antifungal action of these agents alone and in combinations in various vehicles under different experimental conditions. In addition, comparisons were made with products containing fatty acids and fatty acid salts which have been recommended for the treatment of dermatophytoses. The present paper describes the results obtained and includes toxicity data on 3 preparations which have been used in extensive clinical trials in the treatment of various forms of tinea.

The fungicidal efficiency of TCAP is penetrant vehicles against *Microsporon audouini* has been described in a preliminary report by MacKee, *et al.* (13). These authors found that TCAP in an "Intraderm" base was the best fungicidal agent among a large number examined when tests were conducted with infected hairs from cases of tinea capitis. The clinical results obtained with this preparation were gratifying and the course of treatment was well tolerated.

MATERIALS AND METHODS

Liquid culture medium was made with 1% Nutripeptone (Baltimore Biological Laboratories) and 4% maltose. The agar media consisted of this broth plus 2% agar. Three methods of *in vitro* assay have been used to test the antifungal action of the preparations under study.

1. **Fungistatic Tests (Agar or Broth Dilution Method).**—A modification of the Kolmer method in which 0.1 ml. of dilutions of the drugs in water is added to 4.9 ml. of melted agar or broth. The *p*H of the media was adjusted to 5.0 and 7.0 by the addition of MacIllvaine's buffers after sterilizing. Spore suspensions were prepared by washing 1 ten-day agar slant culture of *Trichophyton gypseum* (American Type Culture Collection #9533) *Trichophyton purpureum*, *M. audouini*, *Microsporon lanosum*, *Epidermophyton inguinale*, or *Monilia albicans* in 15 ml. of saline. In the tests 0.01 ml. of the spore suspensions was added to each tube. The results were recorded after two weeks' incubation at 30°. Inhibitory concentrations are expressed as final dilutions per ml. of agar. We have expressed the results obtained by this method as "fungistatic action," although transoculation experiments have proved that the TCAP preparations are fungicidal in all but the highest dilutions.

2. **Fungistatic Tests (Diffusion Zone Method in Agar).**—Ten milliliters of agar inoculated with 0.01 ml. of a heavy spore suspension (*T. gypseum*) is poured into a Petri dish and allowed to harden. Porcelain tops are used to minimize condensation. When liquids are to be tested, 1-cm. filter paper circles are dipped in the preparations, drained, and placed on the surface of the agar. Ointments are placed on the surface of the inoculated agar in 1-cm. circles. Inhibition zones are measured after four days' incubation at 30°.

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3. Fungicidal Tests.—The fungicidal action of liquids was determined by the Emmons (17) and by the Burlingame–Reddish methods (18), using 10-ml. tubes of 1% neopeptone, 2% dextrose broth adjusted to *p*H 7.0 by the addition of sterile phosphate buffer. *T. gypseum* ATCC #9533 was used as the test organism. A special procedure, which we have called the "agar mixture technique," was devised to determine the fungicidal action of ointments. Five-tenths milliliter of the preparation under test was mixed with 9.0 ml. melted Sabouraud's agar (Difco) *p*H 5.6 in a test tube which was then cooled to 45°. The agar was poured into a Petri dish containing 0.5 ml. of a dilution of *T. gypseum* spores, mixed thoroughly, and made to harden quickly by holding the plate for two minutes on a chilled surface. The inoculum was adjusted so that the addition of 0.5 ml. to 9.5 ml. of the agar-drug mixture gave one million spores per ml. of the final agar mixture. Plugs 0.5 cm. in diameter were removed from the agar with a sterile cork borer and seeded into 10 ml. of broth adjusted to *p*H 7 at ten-, twenty-, and thirty-minute intervals after the plates were poured. Results were recorded after two weeks' incubation at 30°.

Composition of Experimental Antifungal Preparations

Experimental ointments were prepared by conventional methods using a partly esterified mixture of fatty acids, principally stearic, but containing oleic and palmitic acids and higher alcohols, as the "wax" component.

The basic formula to which the various active ingredients were added was as follows:

Wax	21.0 Gm.
Spermaceti	3.3 Gm.
Propylene glycol N. F.	5.5 Gm.
Water *q. s.* to make	100.0 Gm.

In order to facilitate discussion and to illustrate the formulation of the experimental ointments in general Formula "A" is given as an example:

Formula "A"

Trimethyl cetyl ammonium pentachlorphenate (40% aqueous slurry)	5.5 (2.2%)
Undecylenic acid	1.0
Wax	21.0
Spermaceti	3.3
Propylene Glycol N. F.	5.5
Water (Distilled)	63.7

Variations of this formula made for comparisons are indicated in Table I.

The solutions studied were variations of the preparation described by MacKee, *et al.* Formula "B" is a fungicidal solution containing 3.2% TCAP, 1% undecylenic acid, 10.8% propylene glycol, 9.8% sulfonated castor oil, 12.6% antipyrine, a wetting agent of the alkyl benzene sulfonate type 5.2%, and water.

Formula "C" is a fungicidal solution containing 1% TCAP, 1% undecylenic acid, 12% propylene glycol, 12% sulfonated castor oil, 3.5% antipyrine, a wetting agent of the alkyl benzene sulfonate type 6%, and water.

All three of the preparations tested had a *p*H of about 5.0.

EXPERIMENTAL

A variety of experimental preparations were compared for antifungal activity by the agar dilution technique using *T. gypseum* as the test organism. The results are shown in Table I.

TABLE I.—ANTIFUNGAL ACTION OF VARIOUS PREPARATIONS DILUTED IN WATER AND INCORPORATED IN SABOURAUD AGAR AT *p*H 5.6 AND *p*H 7 AGAINST *T. gypseum*

	*p*H 5.6	*p*H 7
5% propionic ointment	<1/500[a]	<1/500
1% undecylenic ointment	1/1000	<1/500
5% undecylenic ointment	1/8000[b]	<1/500
5% caprylic ointment	1/3000	<1/500
2.2% TCAP ointment	1/4000[c]	1/1500
(Formula A) 2.2% TCAP + 1% undecylenic ointment	1/4000	1/1500
2.2% TCAP + 5% undecylenic ointment	1/8000[c]	1/1500
2.2% TCAP + 5% caprylic ointment	1/4000	1/1500
TCAP liquid (Formula B) 3.2% TCAP + 1% undecylenic acid	1/8000[d]	1/2500
TCAP liquid (Formula C). 1% TCAP + 1% undecylenic acid	1/3000	1/1000
TCAP Liquid 3.2% TCAP 1% propionic acid (Formula B except propionic replacing undecylenic acid)	1/8000	1/2500
Zinc undecylenate 20% / Undecylenic acid 5% } ointment	1/1500	<1/500
Sodium propionate 16.4% / Propionic acid 3.6% } ointment	<1/500	<1/1500

[a] The figures shown indicate the dilutions of ointment per ml. of agar preventing the growth of *T. gypseum*.
[b] Subcultures of agar show fungicidal action at a dilution of 1/2000.
[c] Subcultures of agar show fungicidal action at a dilution of 1/3000.
[d] Subcultures of agar show fungicidal action at a dilution of 1/5000.

The data in Table I show that the antifungal properties of the various preparations are a function of the concentration of the ingredients and of their behavior at *p*H 5.6 or *p*H 7.

Propionic acid and sodium propionate are known to be weak fungistatic agents and ointments containing large amounts of these ingredients were not inhibitory at a 1/500 dilution. The 1% and 5% undecylenic acid ointments were active at *p*H 5 roughly in proportion to concentration of the acid. The 5% caprylic acid ointment was weaker than the 5% undecylenic acid ointment. The zinc undecylenate ointment was significantly less active than the

5% undecylenic acid ointment. The 2.2% TCAP ointment inhibited when diluted 1/4000. The addition of 1% undecylenic acid or 5% caprylic acid to this formula did not increase its inhibitory action. However, when 5% undecylenic acid was added it inhibited at a dilution of 1/8000. It is of importance to compare the degree of inhibition obtained at *p*H 5 with those obtained at *p*H 7. It will be noted that at the higher *p*H, the preparations containing TCAP were still active at a dilution of 1/1500 or higher, while those containing fatty acids or salts of fatty acids failed to inhibit at the 1/500 dilution.

The activity of the TCAP ointments at *p*H 7 is, therefore, an expression of the antifungal properties of the quaternary ammonium salt. Furthermore, it will be noted that the addition of 1% undecylenic or 5% caprylic acid to ointments containing 2.2% TCAP does not increase their antifungal properties over that of an ointment containing 2.2% TCAP only. When 5% undecylenic acid is added to the 2.2% TCAP ointment, however, the inhibitory titer rises to 1/8000.

The inhibitory titer exhibited by the TCAP liquids at both *p*H 5 and *p*H 7 are what would be expected on the basis of the amounts of TCAP present. It is obvious that the TCAP-undecylenic acid preparations, as exemplified by Formulas A B, and C, have marked antifungal properties.

It has previously been shown that higher end points are obtained when broth is used to test the fungistatic action of fatty acids than when tests are made in agar medium. We have confirmed this observation (24) using the fatty acids and have shown that TCAP is more active when tested in broth than when tested in agar. Recently Quisno, *et al.* (27) have shown that the action of quaternary ammonium salts are impaired in the presence of agar. It was of importance to determine the comparative activity of Formulas A, B, and C, when tested by the dilution method in agar and broth. The results of such a comparison are shown in Table II.

The data in Table II show that an even higher range of activity is manifest by preparations A, B, and C when tested in broth than when tested in agar dilutions. It is probable that the lower fungistatic end points obtained in agar, as compared with those obtained in broth, are due to inhibition of TCAP by agar. However, as previously noted, agents other than quaternary ammonium compounds give higher values when tested against pathogenic fungi in broth than when tested in agar.

Growth inhibition tests were also carried out against *T. purpureum*, *M. audouini*, *M. lanosum*, *E. inguinale*, and *Monilia albicans* by the agar dilution technique. The results are shown in Table III.

The data in Table III show that Formulas A, B, and C are highly active against pathogenic fungi other than *T. gypseum*. All 3 preparations inhibit *T. purpureum*, *M. audouini*, *M. lanosum*, and *E. inguinale* in high dilution when tested at *p*H 5.6.

The preparations are less active when tested at *p*H 7, yet even at this *p*H considerable activity is retained. *Monilia albicans* is more resistant to the drugs than are the fungi.

The inhibiting action of several of the preparations in Table I, as indicated by the growth inhibition zone technique, are shown in Table IV.

It has been previously noted that the width of the inhibitory zones produced by such agents as the higher fatty acids and TCAP is influenced by their solubility (15, 16). This being the case, indications of the comparative antifungal activity of these materials can only be obtained if serial dilutions in suitable solvents are compared (24). When the data in Table IV are compared with those in Table

TABLE II.—ANTIFUNGAL ACTION OF VARIOUS PREPARATIONS DILUTED IN WATER AND INCORPORATED IN AGAR OR BROTH MEDIUM AT *p*H 5.6 and *p*H 7 AGAINST *T. gypseum*

	*p*H 5.6		*p*H 7.0	
	Agar	Broth	Agar	Broth
Formula A, TCAP ointment 2.2% TCAP, 1% undecylenic acid	1/4000	1/6000	1/1500	1/2000
Formula B, TCAP liquid 3.2% TCAP, 1% undecylenic acid	1/8000	1/12,000	1/2500	1/4500
Formula C, TCAP liquid 1% TCAP, 1% undecylenic acid	1/3000	1/4500	1/1000	1/2000

TABLE III.—FUNGISTATIC ACTION OF TCAP-UNDECYLENIC ACID OINTMENT AND LIQUIDS AGAINST VARIOUS PATHOGENIC FUNGI AND *Monilia Albicans*

	Dilutions of Preparations Inhibiting Growth in Agar at *p*H 5.6 and *p*H 7.0					
	Formula A		Formula B		Formula C	
	Agar *p*H 5.6	Agar *p*H 7.0	Agar *p*H 5.6	Agar *p*H 7.0	Agar *p*H 5.6	Agar *p*H 7.0
T. purpureum	1/4000	1/800	1/10,000	1/1000	1/5000	1/800
M. audouini	1/5000	1/800	1/10,000	1/1000	1/5000	1/800
M. lanosum	1/5000	1/800	1/5000	1/800	1/5000	1/600
E. inguinale	1/5000	1/800	1/16,000	1/1000	1/5000	1/800
Monilia albicans	1/400	1/500	1/500	1/400	1/400	1/400

TABLE IV.—INHIBITION OF *T. Gypseum* BY VARIOUS ANTIFUNGAL AGENTS DIFFUSING THROUGH SABOURAUD'S AGAR *p*H 5.6

	Cm.
2.2% TCAP ointment	3.8
2.2% TCAP + 1% undecylenic acid ointment (Formula A)	4.2
2.2% TCAP ointment + 5% undecylenic acid	4.5
Zinc undecylenate 20% / Undecylenic acid 5% } Ointment	4.5
Sodium propionate 16.4% / Propionic acid 3.6% } Ointment	5.4
Liquid (Formula C), 1% TCAP, 1% undecylenic acid (Formula C)	4.8
Liquid (Formula B), 3.2% TCAP, 1% undecylenic acid (Formula B)	5.2

I, it is apparent that no true gauge of the comparative activity of ointments containing water-insoluble antifungal agents can be obtained by merely placing ointments on a culture plate, according to the commonly used inhibition zone technique.

In Table IV, the sodium propionate ointment produced the widest zone of inhibition, obviously because the active ingredient is water soluble. The zone of inhibition produced by this ointment is wider than that produced by the 3.2% TCAP, 1% undecylenic acid solution, yet, the propionate ointment failed to inhibit growth when diluted 1/500 (Table I), while the TCAP-undecylenic acid solution was inhibitory at a dilution of 1/8000 and was proved by subculturing to the fungicidal at a dilution of 1/5000. The fungicidal effects of TCAP solutions were tested by the Emmons technique (17) and by the Burlingame–Reddish technique (18). The results are shown in Table V.

technique. These results are shown in Table V.

It will be seen in Table V that the marked fungicidal activity of the TCAP solutions is demonstrated by the Emmons and the agar mixture technique. It is of interest also to note the fungicidal capacities of the ointments as shown by the agar mixture technique. One to 20 dilutions of the 2.2% TCAP, 1% undecylenic acid ointment, and the zinc undecylenate ointments killed *T. gypseum* spores within ten minutes, but the sodium propionate ointment failed to kill within the thirty-minute exposure period. It was noted above that agar may have an adverse effect on the activity of TCAP, and for this reason the agar mixture fungicidal technique cannot be claimed to give critical quantitative values. In our hands, however, the method has given useful data, and we regard it as an additional tool in studying antifungal drug preparations.

Numerous acute and chronic toxicity studies on TCAP in the Intraderm base were made in animals and sensitization studies were made in rats, rabbits, and guinea pigs repeatedly inuncted with the solution. In addition, large numbers of patch tests and cumulative inunction tests were made in humans to determine primary irritation or hypersensitivity to Intraderm TCAP and TCAP Ointment.

No evidence of primary irritation or sensitization or of chronic toxicity was noted in the inuncted animals, and no instance of irritation or sensitization occurred in a series of 330 human subjects repeatedly inuncted with the products and retested after a two-week interval. The acute toxicity of a 2.5% TCAP solution in water was determined on mice The results, expressed in terms of % survival five

TABLE V.—FUNGICIDAL ACTION OF VARIOUS PRODUCTS AS SHOWN BY SEVERAL METHODS OF TESTING

	Technique by Which Tests Were Conducted		
	Emmons	Burlingame–Reddish	Agar Mixture
Formula B	Killed 10 min.	Killed 30 min.	Killed 10 min.
Formula C	Killed 10 min.	Not killed 30 min.	Killed 10 min.
Formula A			Killed 10 min.
Zinc Undecylenate 20% / Undecylenic Acid 5% } Ointment			Killed 10 min.
Sodium propionate 16.4% / Propionic acid 3.6% } Ointment			Not killed 30 min.
Sodium propionate 16.4% / Propionic acid 3.6% } Liquid	Not killed 30 min.	Not killed 30 min.	Not killed 30 min.

Rapid killing of spores (within ten minutes) was demonstrated by the Emmons method; this result is in keeping with the result of MacKee, *et al.* However, no killing was detected within thirty minutes by the Burlingame–Reddish method.

In order to study the killing effects of the liquids under conditions which would assure contact of the active ingredients with the spores, a special technique, described under Methods and called the "agar mixture technique," was devised. It was feasible to study the fungicidal capacities of ointments by this

days after the subcutaneous injection of the solution are shown in Table VI.

TABLE VI.—ACUTE TOXICITY OF 2.5% AQUEOUS TCAP SOLUTION (MICE)

Subcutaneous Injections, Ml./Kg.	Survival after 5 Days, Per Cent
20	30
15	60
10	100

DISCUSSION

The data presented emphasize the facts previously noted by us that solubilized undecylenic acid and TCAP have remarkable fungicidal properties and indicate that their use in combination according to either Formulas A, B, or C is warranted because of the high order of activity shown *in vitro* (Table I) and because of the absence of contraindications on grounds of toxicity.

We have previously noted (15, 16, 24) the superiority of free fatty acids over the corresponding salts in antifungal activity and have pointed out that TCAP is similarly influenced by *p*H although to a lesser extent since considerable activity is shown by this compound at *p*H 7 and *p*H 8. It should be emphasized that the products containing the combination of active ingredients are extremely active at the *p*H of the normal glabrous skin and scalp and retain much activity even at higher *p*H such as may be encountered in infected areas or in the places where sweat evaporation is retarded (26).

The vehicles described have been prepared on the basis of compatibility with the active ingredients. Particular attention has been given to obtaining a mildly acid *p*H because of superior activity in the range of *p*H of the normal skin.

The use of solubilizing vehicles is important in obtaining the best results (15, 16, 24). It is obvious that water-insoluble active ingredients must be incorporated in suitable solubilizing vehicles if maximum effects are to be obtained. In the case of the TCAP, undecylenic acid ointment (Formula A) the wax combination used forms a solubilizing emulsion in which the active ingredients are dispersed. The liquid vehicles (Formulas B and C) have specialized properties. Formula B has well-defined skin penetrating action (19–23) and has been prepared for the treatment of intractable fungous infections, especially those in which the fungus may be thought to reside below the skin surface.

Formula C contains 1% TCAP and 1% undecylenic acid in true solution and has a high degree of fungicidal action (Table I). It was prepared for the treatment of, and as a prophylactic against, dermatophytoses of the glabrous skin, and as a prophylactic shampoo against tinea capitis.

The data in Table I show that the preparations containing TCAP and undecylenic acid have powerful antifungal action. The ointment (Formula A) containing 2.2% TCAP and 1% undecylenic acid capable of inhibiting the growth of *T. gypseum* when diluted 1/4000 in agar media at *p*H 5.6, the Intraderm liquid (Formula B) containing 3.2% TCAP inhibits at a dilution of 1/8000, and the fungicidal liquid (Formula C) containing 1% each of TCAP and undecylenic acid inhibits at a dilution of 1/3000. Still higher ranges of activity are obtained when tests are carried out in broth, and it is probable that the figures obtained by methods involving the use of agar culture media fail to reveal the true antifungal activity of the products containing TCAP.

A high order of activity is manifest by all 3 preparations at *p*H 7.0. Furthermore, as can be seen in Table IV, they have a rapid fungicidal action. Another point of interest in regard to the physical charactristics of the preparations is seen in Table III, in which a satisfactory degree of diffusion of the active ingredients in agar media is demonstrated.

SUMMARY AND CONCLUSIONS

The antifungal activity of 3 preparations containing trimethyl cetyl ammonium pentachlorphenate (TCAP) and undecylenic acid has been compared with other preparations containing fatty acids or their salts, some of which have been reported to be useful for the treatment of dermatophytosis.

The TCAP-undecylenic acid preparations were found to be more active against pathogenic fungi than were the other preparations studied.

The effect of solubilizing vehicles and *p*H of the preparations on the activity of fatty acids and TCAP has been discussed.

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Stability of Iodine Solutions and Tinctures*,†,‡

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Storage experiments on Iodine Tincture U. S. P., Strong Iodine Tincture N. F., Solution of Iodine N. F., and Strong Solution of Iodine U. S. P. have been carried out over a period of thirty months. These preparations were found to be stable in clear, amber, and blue glass-stoppered bottles. Additional studies on Iodine Tincture when in actual use in first-aid cabinets for a period of a year showed that this preparation maintained the required Pharmacopœia strength. Assay of collected samples of Strong Iodine Tincture which had been subject to household use for indefinite periods indicate that improperly stored samples of this product may have an iodine content higher than that permitted by the official compendia.

ALTHOUGH a tincture of iodine was not recognized in the first Pharmacopœia of the United States, we find that both of the 1830 revisions (1, 2) contain Tincture of Iodine. The Pharmacopœia of 1840 (3) recognized not only Tincture of Iodine prepared by dissolving 1 oz. of iodine in a pint of alcohol, but also a Compound Tincture and a Compound Solution of Iodine. The Compound Solution was prepared by dissolving 6 drachms of iodine and $1^1/_2$ oz. of potassium iodide in a pint of water. This preparation has remained virtually unchanged during the past century and appears in the Thirteenth Revision (4) as Strong Solution of Iodine. The Compound Tincture contained $^1/_2$ oz. of iodine and 1 oz. of potassium iodide in a pint of alcohol, which is approximately half the strength of Strong Tincture of Iodine N. F. VIII (5). In the 1850 revision (6) the Compound Tincture was deleted. The status of the approximately 7 per cent tincture remained relatively unchanged until 1905 at which time the Eighth Revision (7) directed that 5 per cent of potassium iodide be added. U. S. P. IX (8) stated that 50 cc. of water should also be added. This preparation remained in the Pharmacopœia until the Thirteenth Revision at which time it was deleted and is now official in National Formulary VIII (5).

A 2 per cent tincture was added to U. S. P. XI (9) as an antiseptic solution for extemporaneous skin sterilization and for use as a first aid dressing for cuts and wounds in the home. This Mild Tincture was introduced after extensive investigation (10, 11) had shown its general usefulness and bactericidal efficiency. The milder tincture does not appear to destroy tissues thus promoting more rapid healing than does the 7 per cent tincture. In addition, the 2 per cent tincture is prepared with dilute alcohol rendering it considerably less irritating when applied to open wounds. The Pharmacopœial Subcommittee on Scope recognized the advantages of the 2 per cent tincture and voted to delete the 7 per cent tincture and to change the name of Mild Tincture of Iodine U. S. P. XII to Iodine Tincture U. S. P. XIII. Realizing the usefulness and popularity of the stronger tincture the National Formulary VIII (5) adopted the 7 per cent tincture.

Karns, Cretcher, and Beal (12) have demonstrated that protein absorbs iodine more readily from an aqueous solution than from an alcoholic solution of equal strength. Further studies indicate that the dosage deposited depends not only on the solvent, but also on the iodide to iodine ratio. It has been found by adjusting this ratio that a solution could be obtained which would deposit an adequate dosage yet contain a smaller amount of iodine. A 2 per cent aqueous solution proved satisfactory in

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‡ The names of tinctures and solutions used in this paper are in conformance with the new titles adopted for U. S. P. XIII. *The familiar 7% Tincture of Iodine has been deleted* from the U. S. P. and is official as Strong Iodine Tincture N. F. VIII. Mild Tincture of Iodine U. S. P. XII is known as Iodine Tincture U. S. P. XIII. Solution of Iodine has been deleted from the U. S. P. and is official as Iodine Solution N. F. VIII. Strong Iodine Solution (Lugol's solution) remains in the U. S. P.
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‖ Former Fellow, Department of Research in Pure Chemistry, Mellon Institute.
¶ Former Industrial Fellow, Mellon Institute.

this respect. In such a solution the chemical and osmotic contributions to pain are considerably lessened, thereby giving a more physiologically desirable preparation.

A water solution of iodine is superior to an alcoholic solution in rate of penetration (13) and is just as efficient as a bactericide (14). The clinical test of Secor (15) showed the 2 per cent aqueous solution to be an excellent antiseptic for general hospital use. Consequently, Solution of Iodine was made official in the U. S. P. XII (16). However, this product did not gain popular acceptance, possibly because of the low rate of evaporation of the aqueous solvent and the higher freezing point, and was deleted from U. S. P. XIII. It is official under the name Iodine Solution in N. F. VIII.

The stability of tinctures and solutions of iodine has been questioned on many occasions since they were first introduced into the Pharmacopœia. Investigators have claimed that the changes which take place have been due to the glass (17), the impurities in the alcohol (18), the closures (19), heat (20), and light (21). Potassium iodide was used in the preparation of Solution of Iodine and in Compound Tincture of Iodine in the Pharmacopœia of 1840 (3). It is probable that the addition was for purposes of solubility rather than stability but writers soon pointed out the fact that potassium iodide increased the keeping qualities (20, 22, 23). In 1916 Gianturco (20) stated that six months was the maximum safe period for keeping iodine solutions intended for chirurgical purposes. In 1928 Bohrish (21) claimed that the preparations are stable but are kept best in completely filled containers of brown glass. Others (18, 24, 25) have also shown that the preparations are relatively stable.

Since the Thirteenth Revision of the Pharmacopœia (4) recognizes one tincture and one solution and the National Formulary (5) recognizes one tincture and one solution, it was thought it would be interesting to study the keeping qualities of these preparations. We have stored Iodine Tincture U. S. P., Strong Iodine Solution U. S. P., Strong Iodine Tincture, N. F., and Iodine Solution N. F. under various conditions and in clear, amber, and blue glass-stoppered bottles, the light transmission of which has been measured. Our observations are that even when stored under drastic conditions, such as exposure to direct sunlight during summer months, the preparations are relatively stable. The free iodine content of the official preparations showed little change during the thirty-month storage. Measurements of acidity showed that the tinctures after storage ranged from *p*H 3.8 to *p*H 4.7 whereas the aqueous solution measured *p*H 7.0 to *p*H 7.5. Apparently no appreciable acidity is developed during storage.

It is realized that placing the preparations in glass-stoppered bottles and opening them periodically to remove samples for analysis may not be typical of the conditions to which such preparations are subjected in actual use. Since some manufacturers have claimed that the 2 per cent tincture does not maintain its strength when in use, we have filled 2-oz. clear, glass-stoppered bottles with tincture of iodine and placed the bottles in first-aid cabinets where they were in constant use for one year. The use of glass-stoppered bottles eliminated the possibility of reaction with the closure and insured compliance with U. S. P. packaging requirements in that the container shall not interact physically or chemically with the drug it holds.

The re-analysis of these samples after one year showed that there was very little change although in some cases only a few cubic centimeters of tincture remained in the bottle. There was a slight increase in the per cent of free iodine, but none of the samples rose above the permissible maximum limit of 2.2 per cent.

To investigate further the practical keeping qualities of iodine preparations we collected bottles of Strong Iodine Tincture from individuals. These samples had no known history other than that they were purchased by the individual as Tincture of Iodine U. S. P., some of them probably five years previous to our collection. Since no previous analysis had been made by us it can only be assumed that these samples were within the limits for free iodine when

purchased. Our analysis showed that 8 of the 13 samples collected had an iodine content greater than 7.5 per cent, the maximum permitted for the official product. Some of the closures on the samples containing the higher per cent of iodine were faulty, which could account for increase through evaporation of solvent.

Our experiments indicate that the official preparations of iodine are relatively stable when properly stored, i.e., with closures resistant to iodine. The results of the analysis of the household samples of the Strong Iodine Tincture showed that this preparation in actual home use apparently loses alcohol by evaporation, thus increasing its iodine content. Iodine Tincture showed little increase in iodine content when in use in first-aid cabinets during the course of one year, indicating that if this product is stored properly it is a stable preparation.

EXPERIMENTAL

Storage of Official Preparations in Clear, Amber, and Blue Glass-Stoppered Bottles

Iodine Tincture, Strong Iodine Tincture, Iodine Solution, and Strong Iodine Solution were made in accordance with directions given in the United States Pharmacopœia XIII (4) and in the National Formulary VIII (5). The preparations were placed in 2-L., glass-stoppered bottles and allowed to age for a period of three months in the dark. They were then transferred to clear, amber, and blue, glass-stoppered bottles. A detailed description[1] of these bottles follows:

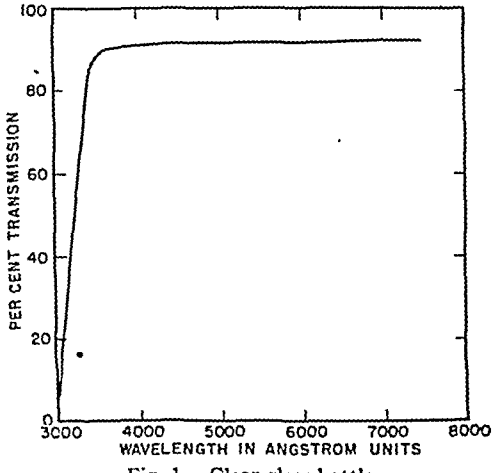

Fig. 1.—Clear glass bottle.

[1] The authors wish to express their appreciation to Mr. J. F. Greene, Director of Research, The Kimble Glass Company, for the curves on light transmission.

Clear Glass Bottles.—These were 16-oz. capacity, square, and glass stoppered. Ten grams of the powdered glass when prepared and treated in accordance with directions given in U. S. P. XIII for Type I Glass containers gave a titration of 15.8 cc. of 0.02 *N* sulfuric acid. Measurements on a sample of the glass 1.9 mm. thick averaged 91% light transmission between wave lengths 4000 Å. to 7000 Å. This curve is shown in Fig. 1.

Amber Glass Bottles.—These were square, 16-oz. capacity, and glass stoppered. Ten grams of the powdered glass when prepared and treated in accordance with directions given in U. S. P. XIII for Type I Glass containers gave a titration of 1.1 cc. of 0.02 *N* sulfuric acid. Measurements on a sample of the glass 1.8 mm. thick showed a range from 2% to 70% light transmission between wave lengths 4000 Å. to 7000 Å. This curve is shown in Fig. 2.

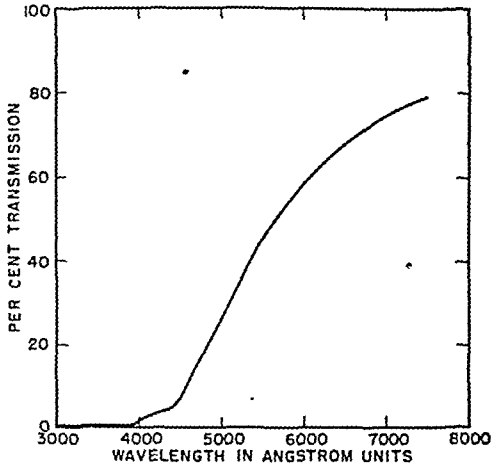

Fig. 2—Amber glass bottle.

Blue Glass Bottles.—These were round, 8-oz. capacity, and glass stoppered. Ten grams of the powdered glass when prepared and treated in accordance with directions given in U. S. P. XIII for Type I glass containers gave a titration of 13.1 cc. of 0.02 *N* acid. Measurements on a sample of the glass 2.2 mm. thick gave an irregular light transmission curve showing 88% transmission at 4000 Å., 64% at 6000 Å., and 90% at 7500 Å. This curve is shown in Fig. 3.

The bottles, filled with preparations to be tested, were placed on a shelf in the laboratory so as to duplicate usual storage conditions. These were assayed at three-month intervals and then transferred to another storage space. At no time were they protected from light in any way. For two separate three-month periods they were placed on a window sill where they were exposed to direct sunlight. One of these periods was during the summer months and on several occasions a thermometer placed near the bottles reached 40°. After

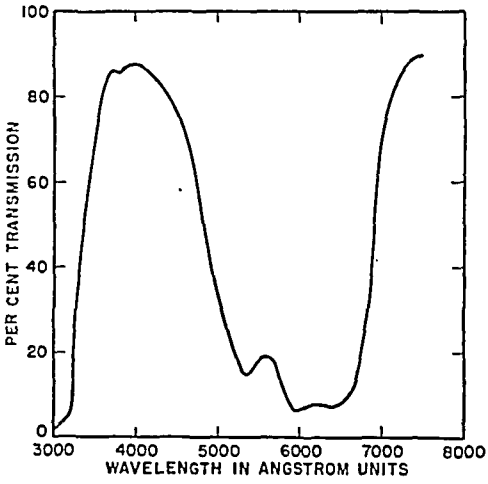

Fig. 3.—Blue glass bottle.

this drastic storage period the Strong Iodine Tincture showed a loss of about 0.5% of its weight; the Iodine Tincture lost about 0.3% in weight; the Iodine Solution lost about 0.3% and the Strong Iodine Solution lost about 0.2%. This loss in weight was probably due to evaporation through the stoppers since the percentage of iodine in all preparations showed a slight increase. If there was a loss of iodine, the loss was less rapid than that of the solvent under such drastic storage conditions.

The per cent of iodine in these preparations over the thirty-month storage period is given in Table I.

Although not recorded in the table, the iodide content of the preparations showed no significant changes. The final *p*H values were determined with a glass electrode. In such unbuffered solutions this figure may be meaningless, but it indicates that no appreciable acidity was developed. It may be observed from Table I that the only significant change in iodine content has occurred in the amber bottle containing the Iodine Solution. The iodine content of this bottle dropped from 1.93% to 1.75%, or a loss of slightly over 9% of the total iodine content. With this possible exception it is noticed that the transmission of light through the container has little effect on the stability of the preparations; the indications are that light is a minor factor influencing the stability. It is believed that the slight differences noticed are not significant in the therapeutic usefulness of iodine preparations. It may be concluded from this study that these iodine preparations when stored according to U. S. P. specifications, in tight containers with iodine resistant closure, may be expected to maintain their required strength for several years. The requirement for light-resistant containers for Strong Iodine Tincture appears to be unwarranted.

Iodine Tincture in Use in First-Aid Cabinets

Four liters of Iodine Tincture were prepared in accordance with U. S. P. XIII instructions and placed in 2-L., clear, glass-stoppered stock bottles. One of the stock bottles was stored in the dark for one year and the other was used to fill 2-oz., clear, glass-stoppered bottles which were placed in first-aid cabinets and subjected to continued use for one year. The only attention given the tinctures during the year was to remove them from the cabinets before they were entirely empty so that some sample would be available for analysis at the end of the year test period. This was necessary in the case of 2 bottles, which contained only 5 to 10 cc. when removed. Table II summarizes the results of this study.

One must conclude that Iodine Tincture is relatively stable under conditions of use. There is a

TABLE I.—STABILITY OF IODINE PREPARATIONS

	Type Bottle	Per Cent Iodine: Freshly Prepared	3 Mo. Aging	6 Mo. Storage	18 Mo. Storage	30 Mo. Storage	Final *p*H
Iodine Tincture U. S. P. XIII	Clear	1.95	1.93	1.94	1.91	1.99	3.8
	Amber	.:	..	1.96	1.94	2.00	3.8
	Blue	..	..	1.95	1.91	1.99	3.9
Strong Iodine Tincture N. F. VIII	Clear	6.94	6.86	6.92	6.90	7.04	4.7
	Amber	..	..	6.92	6.88	7.06	4.7
	Blue	..	..	6.92	6.93	6.91	5.4
Iodine Solution N. F. VIII	Clear	1.95	1.93	1.93	1.89	1.90	7.5
	Amber	..	..	1.83	1.78	1.75	7.0
	Blue	..	..	1.89	1.84	1.82	7.5
Strong Iodine Solution U. S. P. XIII	Clear	4.95	4.91	4.94	4.89	4.98	7.5
	Amber	..	..	4.95	4.89	4.99	7.5

TABLE II.—IODINE TINCTURE IN FIRST-AID CABINETS

	Iodine %	NaI, %	*p*H
Freshly prepared	2.00	2.20	6.6
Stock sol. after 1 yr.	1.99	2.20	4.0
Bottle 1 after 1 yr.	2.14	2.37	3.9
Bottle 2 after 1 yr.	2.06	2.29	4.0
Bottle 3 after 1 yr.	2.01	2.26	3.9
Bottle 4 after 1 yr.	2.06	2.30	4.0
Bottle 5 after 1 yr.	2.01	2.36	4.0

slight increase in both iodide and iodine content. This is probably due to evaporation of solvent. The *p*H of the solution shows that no appreciable acidity is developed. Although the freshly prepared solution is given as *p*H 6.6, it is normal for such solutions to drop to *p*H 5 within twenty-four hours after preparation. These results are contrary to the fact that a 2% Iodine Tincture is sometimes found on the market with a low iodine content. The loss of iodine in such cases is no doubt due to the absorption by iodine reactive closures.

Studies on Old Strong Iodine Tincture Samples

Thirteen bottles of Strong Iodine Tincture were collected from individuals and analyzed. These samples have no known history other than that they were purchased by the individuals as Tincture of Iodine U. S. P. (labeled in accordance with U. S. P. XI or XII) some probably five years previous to our collection. Since no previous analysis had been made, it can only be assumed that the samples were originally within the limits set by the U. S. P. Some of the closures were faulty, and some labels showed evidence of considerable age. Results of these analyses are shown in Table III.

TABLE III.—ANALYSIS OF OLD STRONG IODINE TINCTURE SAMPLES

No.	Iodine, %	KI, %
1[a]	14.5	16.5
2	7.4	5.6
3	9.3	9.7
4	7.1	5.2
5	7.3	5.7
6	7.1	5.6
7	10.7	10.8
8	8.1	7.4
9	8.2	7.7
10	6.9	5.4
11[a]	16.3	14.8
12	7.7	5.9
13	8.6	7.2

[a] Closures in bad condition.

It may be observed that 8 of the 13 samples exceed 7.5% iodine, whereas 11 of the 13 samples exceed 5.5% potassium iodide, the maximum limits set by the official compendia. No significance can be attached to these figures other than that of actual analyses of household-used Tincture of Iodine. The samples which were found to be extremely high, e.g., Nos. 1, 7, and 11, were obviously very old and showed visible evidence of evaporation. Improperly stored samples such as these have probably given rise to the belief that aged Strong Iodine Tincture is stronger and therefore is harmful.

SUMMARY AND CONCLUSIONS

1. It has been shown that the official iodine preparations when stored in all-glass containers are stable preparations.

2. Clear, amber, and blue bottles are equally satisfactory for storage, showing that light alone has little effect on the stability of the preparations.

3. Iodine Tincture (Mild Tincture of Iodine U. S. P. XII) in proper containers is not subject to appreciable change under actual conditions of use for a period of a year.

4. A survey of household samples of Strong Iodine Tincture has shown that these samples have a tendency to be somewhat stronger than official specifications. This tendency is probably due to improper closures.

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A Modified Kober Method for the Determination of Alpha- and Beta-Estradiol*

By JONAS CAROL and J. C. MOLITOR

A method is described for the determination of α- and β-estradiol by a modified Kober procedure. It is based on the fact that β-estradiol reacts with Kober's reagent in the cold while α-estradiol does not. The results of analysis of a series of samples containing the two isomers are given.

EXISTING chemical methods for the determination of estradiol are of limited value in estimating biological potency since they do not indicate the relative amounts of the α- and β-isomers present. Doisy, Thayer, and Van Bruggen (1) and Pearlman and Pearlman (2) have developed differential bioassays for α- and β-estradiol but these methods require too much time for general analytical use, and are subject to occasional serious error. Whitman, Wintersteiner, and Schwenk (3) have separated the α- and β-estradiols by precipitating the insoluble α-estradiol digitonide. This method was not applicable to samples containing microgram quantities of estradiols. Attempts to separate the isomers by absorption chromatographic methods similar to those used by Stimmel (4) for the separation of estrone, estradiol, and estriol failed as did attempts to make the separation by partition chromatography.

The method of analysis presented in this paper is based on the fact, not previously reported, that in the initial step of the Kober reaction (5) β-estradiol reacts with the reagent at room temperature, while α-estradiol[1] does not. Under these conditions completion of the reaction produces the normal magenta colored solution with the β-isomer and a colorless solution with the α-isomer. By use of this modified procedure, α-estradiol is determined as the difference between total estradiols and β-estradiol. For simplicity of presentation determinations made with the initial reaction carried out at the temperature of boiling water will hereafter be referred to as procedure *A*, and those made with the initial reaction at room temperature will be referred to as procedure *B*. In both procedures, the remainder of the reaction is carried out essentially as directed by Venning, *et al.* (6).

* Received Jan. 31, 1947, from the Medical Division, Chemical Section, Food and Drug Administration, Federal Security Agency, Washington, D. C.

[1] Estrone, estriol, and equilenin, like α-estradiol, do not react with Kober's reagent at room temperature. A very small sample of equilin obtained by the writers produced a bright yellow color in both the initial and final phases of the reactions. No further work has been done to determine if this is a typical color or if it is formed by an impurity. We are indebted to Dr. Oliver Kamm, of Parke, Davis & Company, who supplied us with the samples of equilin and equilenin.

EXPERIMENTAL

Apparatus.—A spectrophotometer or photometer equipped to measure extinction at 420, 526, and 528 mμ. (A Beckman Quartz Spectrophotometer fitted with matched 1-cm. absorption cells was used in this investigation.)

Reagents.—α-Estradiol, m. p. 175–176°, standard solution prepared in 95% alcohol containing 100 micrograms per ml.

β-Estradiol, m. p. 220–222°, standard solution prepared in 95% alcohol containing 50 micrograms per ml.

Kober's Reagent—Slowly add, with mixing, 5.6 parts (wt.) of C. P. grade sulfuric acid to 3.6 parts (wt.) of freshly redistilled phenol. Cool to prevent temperature rise.

Dilute sulfuric acid 25% *v/v*.

Determination of the optimum time for completion of the initial phase of the reaction of β-estradiol in procedure *B* was necessary as a preliminary to the remainder of the investigation. In a series of determinations made with 50-microgram samples of β-estradiol, the time of the initial phase of the reaction was increased by fifteen-minute intervals. The extinction values of the resultant solutions, measured at 523 mμ, and shown in Table I, indicate that the initial reaction is completed within fifteen minutes. Further increases in reaction time result in a gradual loss in color. In the following work an initial reaction period of twenty minutes was used in procedure *B*, so as to conform with that specified by Venning, *et al.* (6), and used in procedure *A*.

The absorption spectra of the solutions formed in procedure *A* with 100-microgram samples of α- and β-estradiol, and the absorption spectrum of the solutions formed in procedure *B* with β-estradiol, were measured from 420–580 mμ. The curves shown in Fig. 1 show that while the absorption spectrum of the color formed by the α-isomer is essentially the same as reported by Venning, *et al.* (6), with a maximum at 523 mμ, the absorption spectra of the

TABLE I.—VARIATION OF EXTINCTION VALUE WITH TIME OF INITIAL REACTION OF SOLUTIONS PREPARED FROM β-ESTRADIOL BY PROCEDURE *B*

Time in Min.	$E_{528m\mu}$, 1 cm.[a]
15	0.323
30	0.314
45	0.295
60	0.285
75	0.272
90	0.263
105	0.259
120	0.248

[a] These measurements were made before it was found that the maximum for the solution formed with β-estradiol is at 528 mμ.

color formed by the β-isomer in both procedures *A* and *B* have maxima at 528 mμ; and that the extinction coefficient at 528 mμ of the product formed with β-estradiol by procedure *B* is about 60% greater than that formed by procedure *A*.

The validity of Beer's law when applied to spectrophotometric measurements made on products formed by β-estradiol in procedure *B*, with and without added α-estradiol, was determined. Two series of solutions were prepared, one from 10–50-microgram samples of β-estradiol, and the other from 10–50-microgram samples of β-estradiol each containing 100 micrograms of added α-estradiol. Extinction measurements were made on these solutions at 528 mμ and these data are recorded in Table II.

A plot of these extinction values against concentration of β-estradiol, as shown in Fig. 2, establishes adherence to Beer's law and indicates no interference from added α-estradiol.

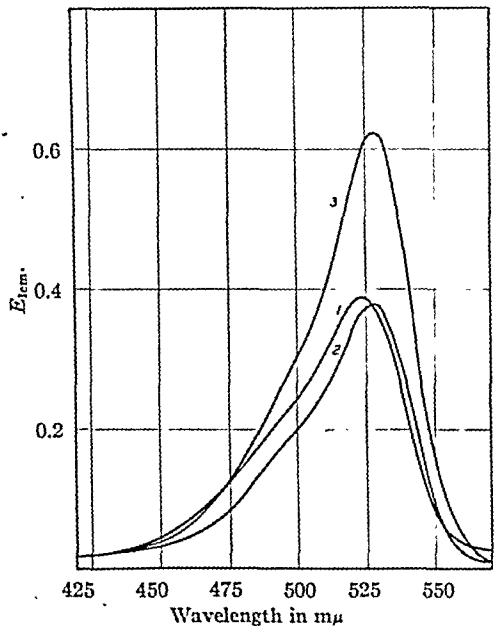

Fig. 1.—The absorption spectra of the colors formed from 100 microgram samples of α- (curve 1) and β- (curve 2) estradiol by procedure a, and β-estradiol (curve 3) by procedure b.

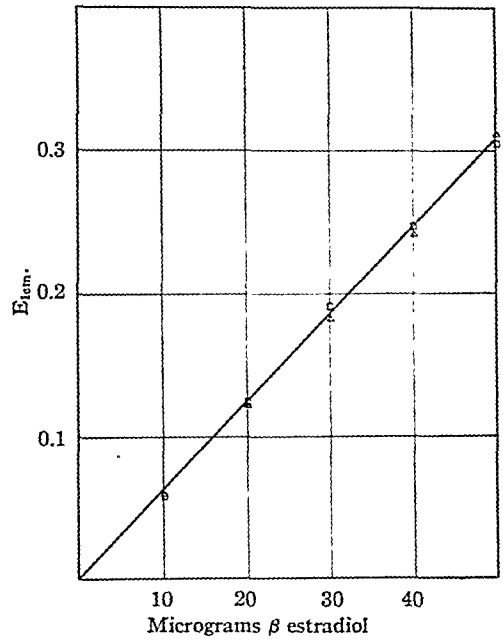

Fig. 2.—Plot of E against concentration of β-estradiol at 528 mμ. Δ without added α-estradiol. □ with 100 micrograms added α-estradiol.

TABLE II.—APPLICABILITY OF BEER'S LAW TO PRODUCTS FORMED BY β-ESTRADIOL IN PROCEDURE *B*

β-Estradiol, Micrograms	$E_{1\,cm.\,528m\mu}$ Without Added α-Estradiol	With Added α-Estradiol
10	0.059	0.053
20	0.118	0.121
30	0.179	0.190
40	0.237	0.246
50	0.310	0.302

It is evident from a study of the absorption data in Fig. 1 that extinction measurements made at the maximum for the α-estradiol color may not give correct results for total estradiols when an appreciable amount of β-estradiol is present. Determinations of total estradiols made from extinction measurements at 526 mμ, the region of equal extinction for both the α- and β-estradiol color (by procedure *A*) should give correct results regardless of the proportion of α- and β-estradiol in the sample. A series of solutions was prepared, by procedure *A*, from mixtures of estradiols varying from 100% of α to 100% of β. Extinction readings of these solutions were made at 526 mμ and the amounts of total estradiols were calculated using α-estradiol as a standard. These results are presented in Table III.

Method.—To each of two 20 × 150 *mm.* test tubes transfer aliquots of an alcoholic solution of the sample containing about 25–100 micrograms of total estradiols. Transfer 1 ml. of α-estradiol standard solution to a third test tube, and 1 ml. of β-estradiol standard solution to a fourth tube. Evaporate each to dryness on a steam bath with aid of a

TABLE III.—RESULTS OF THE DETERMINATION OF TOTAL ESTRADIOLS BY EXTINCTION READINGS AT 526 mμ

Sample	α-Estradiol Micrograms	β-Estradiol Micrograms	$E_{1\,cm.\,526m\mu}$	Total Estradiols Found, Micrograms
1	100	0	0.369	...
2	80	20	0.363	98.4
3	60	40	0 364	98.6
4	40	60	0.369	100.0
5	20	80	0.364	98.6
6	0	100	0.352	95.7

current of air. Dry the tubes in a vacuum desiccator over sulfuric acid for one hour.

Procedure *A*.—To one of the sample tubes, the α-estradiol standard tube, and a blank tube, add 3 ml. of Kober's reagent (from a burette), stopper with tin foil covered corks, and heat in a boiling-water bath for exactly twenty minutes. Mix each tube twice during the first ten minutes of the heating period by shaking vigorously. At the end of twenty minutes, transfer to an ice bath and cool for at least five minutes. Allow tubes to remain in ice while 3 ml. distilled water is added from a burette to each test tube and the contents are thoroughly mixed with a glass rod. Leave the rods in the test tubes for the remainder of the reaction. Place the tubes again in the boiling water bath and heat for exactly three minutes. At the end of three minutes transfer the tubes again to the ice bath and cool for at least five minutes. After cooling, transfer the contents of each tube to a glass-stoppered graduated cylinder and dilute to 15 ml. with dilute sulfuric acid.[2] Mix well and determine the extinction of both the sample solution and α-estradiol standard relative to the blank solution at 420 mμ[3] and 526 mμ.

Micrograms total estradiols in aliquot =

$$\frac{E_{526\ m\mu\ sample} - \frac{E_{420\ m\mu\ sample}}{2}}{E_{526\ m\mu\ standard} - \frac{E_{420\ m\mu\ standard}}{2}} \times 100$$

Procedure *B*.—To the other sample tube, the β-estradiol standard tube, and a blank tube, add from a burette 3 ml. Kober's reagent and mix each tube thoroughly with a glass rod. Leave the rods in the tubes for the remainder of the reaction. Allow the tubes to stand at room temperature for twenty minutes, stirring every five minutes. At the end of twenty minutes, transfer to an ice bath and cool for at least five minutes. Allow tubes to remain in ice while 3 ml. distilled water is added from a burette to each test tube and the contents are thoroughly mixed with a glass rod. Place the tubes in a boiling-water bath and heat for exactly three minutes. At the end of three minutes transfer the tubes again to the ice bath and cool for at least five minutes. After cooling, transfer the contents of each tube to a glass-stoppered graduated cylinder and dilute to 15 ml. with dilute sulfuric acid. Mix well and determine the extinction of both the sample solution and the β-estradiol standard relative to the blank solution at 420 mμ and 528 mμ.

Micrograms β-estradiol in aliquot =

$$\frac{E_{528\ m\mu\ sample} - \frac{E_{420\ m\mu\ sample}}{2}}{E_{528\ m\mu\ standard} - \frac{E_{420 m\mu\ standard}}{2}} \times 50$$

α-estradiol = total estradiols − β-estradiol

A series of mixtures of α-and β-estradiol was prepared and analyzed by the above method. The results of analysis are shown in Table IV.

TABLE IV.—RESULTS OF ANALYSIS OF KNOWN MIXTURES OF α-AND β-ESTRADIOL

Sample	α-Estradiol Micrograms Added	α-Estradiol Micrograms Found	β-Estradiol Micrograms Added	β-Estradiol Micrograms Found
1	5	4.2	100	99.5
2	10	9.0	90	88.1
3	20	24.9	20	20.1
4	20	21.5	80	76.6
5	30	33.4	30	30.4
6	40	41.0	25	27.0
7	40	43.6	40	38.4
8	50	44.0	50	50.2
9	60	57.9	40	40.1
10	75	70.0	35	38.0
11	80	79.2	20	19.7

SUMMARY AND CONCLUSIONS

1. A modified Kober procedure is described for the determination of α- and β-estradiol.

2. Results of analysis of a series of mixtures of α- and β-estradiol are given. Good results are obtained with mixtures containing as little as 5 micrograms of one isomer in the presence of 100 micrograms of the other.

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[2] The color of solutions prepared by the writers according to Venning, *et al.* (6), faded too rapidly to permit accurate colorimetric measurements. Increasing the concentration of the sulfuric acid used to dilute the reaction mixture from 10% to 25% v/v produced colors that remained stable at room temperature.

[3] This correction is based on the findings of Venning, *et al.* (6), Bachman and Pettit (7), and Stimmel (4) that $E_{420m\mu}/E_{526m\mu}$ for nonestrogenic impurities is approximately 0.5.

Availability of Penicillin from Various Ointment Bases*

By H. A. CLYMER and R. J. FERLAUTO

The release of penicillin from various type ointment bases by methods which are believed to simulate closely actual *in vivo* conditions has been investigated. Data from the methods employed give actual concentration figures of the amount of penicillin released by the various type bases. Results obtained are proposed as the basis for a more rational means of choosing bases for penicillin to be used topically.

DURING the past ten years the term *topical application of ointments* has broadened in meaning both as to actual therapeutic intent and as to the type of bases employed. For centuries this implied empirical application by inunction of medicaments contained in fatty bases and was concerned primarily with the treatment of superficial skin disorders or with the attempt to obtain systemic absorption by penetration. With the advent of the newer, more specific, and less toxic antibacterial agents, the practice has become accepted of applying these substances directly to the tissues as anti-infective agents in the treatment of wounds, burns, and in minor surgery. Thus can be achieved the objectives of topical application in attaining high tissue concentrations of the drug at the site of the infection in shorter periods of time and with less drug than by systemic administration. While this change in therapeutic intent has been taking place, advancement has also been made in the bases to be used as vehicles. This has been primarily due to the development of synthetic emulsifying agents, making possible easy preparation of a wide variety of water miscible oil-in-water emulsions. Chamings (1) in a recent address traces this same development of ointment medication from the days of "empiricism" through the present state of experimentation and stresses that the ultimate aim of efficient ointment therapy will only be gained by intelligent choosing of the base from the types now available after thorough consideration of the therapeutic end. In view of these changes, it is no longer simply a matter of placing the new therapeutic agent into Simple Ointment U. S. P. in order to formulate the topical form required. This last statement should be self-evident, but the incidence of this type of formulation of topical therapy has been so frequent the authors feel reiteration to be mandatory.

There has been considerable effort on the part of the pharmaceutical and medical professions to set up specifications for vehicles and bases for topical application. These listings cover requirements ranging from chemical inertness to the cosmetic niceties. They serve as excellent check lists for the compounder when contemplating topical medication. One must not lose sight, however, of the fact that the primary and indispensable quality of a carrier is that it allows adequate release of the active therapeutic medication. The lack of this quality nullifies whatever other value the base may possess. It has been pointed out (2, 3) that many apparent therapeutic failures with sulfonamides were due to improper topical application of the drug with resulting low local tissue concentrations of the drug. In order to avoid a repetition of this type of failure, it is doubly important with the topical use of penicillin that adequate concentrations are maintained, for Molitor (4) has warned that insufficient tissue concentrations create the possibility of pathogenic organisms' acquiring resistance to penicillin. This problem of acquired drug resistance of pathogenic organisms makes the use of preparations affording inadequate release of penicillin not just wastefully ineffective but potentially dangerous.

The present status of bases for penicillin ointment is unsettled. In this country penicillin ointment intended for sale in interstate commerce is limited by the F.D.A.

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Regulation Sec. 146.26 (5) to all grease bases composed primarily of wool fat, petrolatum, or white petrolatum, or any mixture of two or all of these, the use of certain other fats and waxes being optional. The new Addendum (6) to the British Pharmacopœia includes official monographs for an all grease preparation and two oil-in-water emulsion creams. The final choice of bases for topical application of penicillin, as with other medicinal agents, will have to come from elaborate, controlled clinical investigations; reports of such investigations, however, are meager and inconclusive. It is important for sound clinical evaluation to possess a preliminary idea, obtained from adequately controlled *in vitro* tests, of the inherent properties of the medicinal substances when incorporated into the various bases. The lack of this type of information prompted the present investigation.

EXPERIMENTAL PLAN

In any *in vitro* evaluation of antiseptics the basic objective must be to simulate as closely as possible the conditions under which the preparation is to be used *in vivo*. We have limited our testing to that phase of topical application whereby anti-infective agents are applied directly to the tissue, as in the treatment of wounds and burns, and in minor surgery and have not been concerned with application by inunction to intact skin. The following factors were, therefore, considered in choosing the methods to be used:

(*a*) epidermal layer of the skin has been removed, exposing serous tissue fluid, and

(*b*) penicillin concentrations once achieved are not static since tissue fluids are constantly changing

The standard agar diffusion method (7) for testing antiseptic ointments was considered grossly inadequate. Even though appreciable zones of inhibition are obtained with both water-miscible and water-immiscible bases by this method the results indicate only that a sufficient amount of the drug has been made available to produce the inhibition zone under the conditions of the test. Information cannot be obtained as to the quantity or continuity of release of the drug from the base. The method employed by Waud and Ramsay (8) and by Huyck, *et al.* (9) to determine the availability of sulfonamides in topical form is a modification of the agar diffusion test employing chemical indicator rather than bacterial inhibition. The method of Bandelin and Kemp (3) is deemed superior in that it gives some idea of the quantitative release of the drug, but it allows for a variance in surface area and does not simulate change of tissue fluid.

Simulation of *in vivo* conditions to obtain data on the continuity and quantity of release of penicillin was thought to be met best by the following method: Ointments were enclosed in cellophane bags to facilitate repeated transfer of the ointment bulk from first diffusion fluid to successive, fresh fluids and determining the amount of penicillin released in each diffusate. Information as to the continuity of release was adequately obtained by this method. When data so obtained were interpreted as to accurate quantitative release, however, the following shortcomings were observed: rigid control of surface area was impossible, the volume of diffusion media needed was quite large, and the cellophane bag set up a possible barrier which is not present *in vivo*. An improved method was developed whereby the ointment was contained in a shallow glass cup, affording a uniform surface area and allowing a minimum of diffusion media. Serum was substituted for saline, since it more closely simulates tissue fluid. The practice of transferring the ointment from the first diffusion fluid to successive, fresh fluids and determining the amount of penicillin released in each diffusate was again employed.

In choosing the bases to be used in this evaluation, we have confined ourselves mainly to the all grease bases as representative of water-immiscible vehicles, and to oil-in-water emulsions as representatives of water-miscible bases. Water-in-oil emulsions were not used extensively since from preliminary experimentation the release was found to be of the same magnitude as from all grease. The water-soluble base, Carbowax, although showing excellent liberation, was deemed impractical because of the relative instability of penicillin in this compound.

EXPERIMENTAL

The petrolatum,[1] petrolatum-plus-lanolin,[2] and oil-in-water emulsion[3] bases were prepared and then autoclaved. Upon cooling, sufficient calcium penicillin[4] was incorporated so that the finished ointment would contain approximately 1000 u./Gm. An original assay for actual penicillin content was performed on each ointment immediately prior to use.

Continuity of Release (Cellophane Bags).—Visking tubing with an inflated diameter of 1.9 cm. was cut in lengths of approximately 10 cm. One end was tied and then placed in boiling water for three minutes. After draining, 4 Gm. of the ointment to be used was placed into each bag and sealed. The bags were suspended in test tubes, 2.5 cm. x 20 cm., containing 15 cc. of sterile normal saline solution. The bags were weighted so that they remained completely immersed in the saline. The test tubes were closed with rubber stoppers and placed at 37° in an incubator on a rotary agitator so that a gentle swirling action was obtained. After the diffusion

[1] White Perfecta, Sonneborn.
[2] 10% Anhydrous Lanolin, U. S. P.; 90% Petrolatum, U. S. P. (White Perfecta, Sonneborn).
[3] Sodium Lauryl Sulfate 1%. Cetyl Alcohol 10%. Mineral Oil 20%. Glycerin 10%. Distilled Water 59%.
[4] Pfizer, Lot 1955C.

period, the bags containing the ointment were lifted out of the saline solution and allowed to drain in the tube. They were then immediately transferred and immersed in a new tube containing a fresh 15-cc. portion of normal saline for the next diffusion period. This procedure was carried out for four successive diffusion intervals of 3, 3, 16, and 8 hours respectively. The tubes of saline after the diffusion periods were stored under refrigeration until they were assayed for penicillin content.

Constant Surface Area (Shallow Cup).—Each of the samples of ointment to be tested was placed in a containing glass cup with an internal diameter of 3.5 cm. and an internal depth of 0.3 cm. These cups were specially constructed to insure uniform diameter and provided a constant surface area of 9.6 sq. cm. Since the surface area of each of the ointments was to be the same, great care was taken to obtain a smooth continuous level surface of the ointment in the cup. The cup containing the ointment was then placed in a porcelain dish of slightly larger dimensions, i.e., diameter 4.5 cm. and depth of 1.2 cm. Seven cubic centimeters of human serum[5] were added to cover the ointment surface completely, taking care not to disturb the surface of the ointment. The cups were then placed at 37° in an incubator and covered to minimize evaporation. At the end of the diffusion period the inner cup containing the ointment was carefully removed and serum allowed to drain into the outer cup. The cups were gently passed through sterile distilled water to remove adhering serum and then transferred to new outer cups and 7 cc. of serum added. This procedure was repeated for four successive diffusion periods of 8, 2, 2 and 2 hours respectively. The serum diffusates were refrigerated (5°) until assayed for penicillin content.

The assays for penicillin content of the ointments and diffusates were carried out as follows: petrolatum-plus-lanolin ointments were assayed by the F.D.A. agar diffusion method (10) for the determination of potency of penicillin ointments. Since an oil-in-water emulsion is water miscible, it was uniformly dispersed with mechanical agitation in phosphate buffer (*p*H 6) and then assayed by the standard F.D.A. method for penicillin solutions. Assays for penicillin content of the diffusates were carried out employing the standard F.D.A. agar diffusion method for penicillin solutions. Where penicillin concentrations were high the diffusates were diluted to approximately 1 u./cc. of penicillin with phosphate buffer (*p*H 6).

RESULTS

Results from the principal experiments described above are summarized in Tables I and II.

Control tests were performed in the following manner. First, the rates of inactivation of penicillin in serum and saline under the conditions of the test were determined and are recorded as the controls in Tables I and II. The rate of inactivation of penicillin in the serum after eight hours appears exces-

TABLE I.—CONTINUITY OF RELEASE OF PENICILLIN FROM TWO OINTMENT BASES CONTAINED IN CELLOPHANE BAGS[a]

		Ointment Potency, u./Gm.	Diffusion Periods: I 3 Hr., u./cc.	II 3 Hr., u./cc.	III 16 Hr., u./cc.	IV 8 Hr., u./cc.
Petrolatum	#1	895	0.2	0.14	0	0
	#2	895	0.27	0.13	0	0
O/W Emulsion	#1	1260	52.0	19.8	37.5	9.0
	#2	1260	56.0	18.6	35.0	10.3

[a] Control—Calcium penicillin in normal saline lost approx. 8–9% in 16 hr. at 37°.

TABLE II.—COMPARATIVE RELEASE OF PENICILLIN FROM VARIOUS OINTMENT BASES EMPLOYING THE SAME SURFACE AREA[a]

		Ointment Potency, u./Gm.	Diffusion Periods: I 8 Hr., u./cc	II 2 Hr., u./cc.	III 2 Hr., u./cc.	IV 2 Hr., u./cc.
Petrolatum	#1	820	0.184	0	0	0
	#2	820	0.155	0	0	0
Petrolatum Lanolin	#1	810	0.163	0	0	0
	#2	810	0.175	0	0	0
O/W Emulsion	#1	890	75.5	17.8	10.8	9.4
	#2	890	73.5	14.6	11.4	11.3

[a] Control—Stability of calcium penicillin in human serum[b]

Original Potency (50 u/cc.)	% Activity Remaining After 2 hr.	8 hr.
100%	92	40

[b] The serum for this experiment was prepared aseptically by collecting blood directly from the vein, into a bottle containing mineral oil. It was then centrifuged under paraffin and the serum withdrawn and kept layered with mineral oil until used.

sive, serum *p*H, however, had risen to 8.8 by the end of the diffusion period. Serum containing penicillin with a protective layer of mineral oil to prevent carbon dioxide loss remained practically unchanged in *p*H and the rate of inactivation of penicillin was considerably less. In the second control measure, each ointment base was placed in serum and saline containing penicillin under the conditions of the test; the presence of these bases caused no increase in the rate of penicillin inactivation.

DISCUSSION

Analysis of the diffusates after contact with the ointment shows that penicillin in an all-grease base is relatively unavailable to serum or saline. There was a small immediate release but none thereafter, indicating that only the penicillin on the surface of these ointments was available and the bulk of the penicillin remained locked in the base. Release of penicillin from the oil-in-water emulsion bases, however, was high in the first diffusion period, leveling off to a constant high concentration throughout the entire period of testing. The physical nature of the oil-in-water emulsion base undoubtedly is responsible for this effect since the continuous phase of the emulsion is miscible with the surrounding fluid media and allows diffusion to take place. These results parallel what has been found with the sulfonamides.

In order to determine from the standpoint of penicillin stability the feasibility of using the oil-in-water emulsion base, the stability under refrigerated conditions, i.e., 5°, was tested and the results show that 80 per cent of the original penicillin content is available after one month's storage and 50 per cent after two months' storage. This indicates that oil-in-water emulsion is satisfactory for use in compounding extemporaneous penicillin ointments. It must be stressed, however, that the stability is for only the oil-in-water emulsion base used in this experiment, and that any modification would necessitate rechecking to determine the effect upon stability of the penicillin.

SUMMARY

Two *in vitro* methods for the estimation of the amount and continuity of release of penicillin in various ointment bases are described. By these methods an evaluation of penicillin availability from representative water-miscible and water-immiscible bases has been obtained. The quantity of penicillin released was much greater with the oil-in-water emulsion than with the all-grease base. No continuity of release was found with the all-grease base from which there was only a small immediate release and none thereafter. Water miscible oil-in-water emulsion base, however, showed a high continuous release. It is concluded that the use of water-miscible oil-in-water emulsion bases are superior in making penicillin available to tissue fluids.

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Some Minor Alkaloids of Pei-Mu, Fritillaria Roylei*

By T. Q. CHOU†

Four minor alkaloids in addition to the two principal ones previously reported have been isolated from the Chinese drug, Pei-Mu. The procedures employed are described and the analytical data and derivatives used in the characterization of these new alkaloids are reported.

IN A PREVIOUS communication, Chou and Chu (1) reported the preparation and properties of peimine, $C_{26}H_{43}O_3N$ and peiminine, $C_{26}H_{41}O_3N$, the two principal alkaloids of the Chinese drug, Pei-Mu, identified as *Fritillaria Roylei*. From their mother liquor, there have been isolated up to present 4 other alkaloids which are named peimisine, peimiphine, peimidine, and peimitidine, respectively. Their compositions, melting points, and specific rotations are given in Table I.

TABLE I

Name	Formula	M. P., °C.	Specific Rotation
Peimisine	$C_{27}H_{43}O_4N$	270	$[\alpha]_D^{25}$ −51° in alcohol
Peimiphine	$C_{27}H_{46}O_3N$	127	$[\alpha]_D^{21}$ −69° in alcohol
Peimidine	$C_{27}H_{45}O_2N$	222	$[\alpha]_D^{26}$ −74° in alcohol
Peimitidine	$C_{27}H_{44}O_3N$	188	$[\alpha]_D^{20}$ −68° in alcohol

All these alkaloids are found in the drug only in very small quantities, ranging from 0.001 to 0.002 per cent. Their isolation has been effected by taking advantage of the difference in the solubilities of their well-crystallized hydrobromides or hydrochlorides. Like peiminine, peimisine contains in its molecule a carbonyl group, forming easily an oxime, m. p. 196°.

EXPERIMENTAL

Peimisine, $C_{27}H_{43}O_4N$.—In the isolation of peimine and peiminine as reported previously (1), 70 Kg. of the Chinese drug, Pei-Mu, were used. After the removal of these 2 principal alkaloids in the form of their hydrochloride as much as possible by fractional crystallization, all the mother liquors are united and distilled nearly to dryness and the residue taken up with water in which much resinous matter remains insoluble. The aqueous solution is filtered, made alkaline with sodium carbonate, and the precipitate extracted with ether. The ethereal solution, when dried and distilled, leaves behind about 10 Gm. of a basic residue which is dissolved in acetone and neutralized with an alcoholic hydrochloric acid. On standing for a few weeks at room temperature, there separates out a crystalline deposit weighing about 1.5 Gm. and consisting of a mixture of peimisine, peimiphine, and peiminine hydrochlorides. After filtration, it is warmed on water bath for some time with 10 cc. of 93% alcohol in which about half the quantity remains insoluble The insoluble part which consists of the peimisine hydrochloride in an almost pure state is filtered and dried, its mother liquor being reserved for working up peimiphine. It is dissolved in about 70 cc. of boiling water. The aqueous solution, when cooled to room temperature, is made alkaline with sodium carbonate and the liberated base extracted with chloroform. Peimisine is recovered from the chloroform solution by distilling off the solvent and crystallizing the residue from a little alcohol. It forms colorless rhombic prisms, m. p. 270°, $[\alpha]_D^{25}$ −51. It is easily soluble in alcohol and chloroform, but only sparingly soluble in acetone or ether. Its hydrochloride, prepared by neutralizing the pure alkaloid with hydrochloric acid in alcohol, forms prismatic needles, m. p. 257°, and is soluble in water or alcohol with difficulty. Its aurichloride is obtained as an amorphous powder by precipitation in aqueous solution in the presence of hydrochloric acid. The analytical data obtained with the alkaloid, its hydrochloride, and aurichloride agree with the formula $C_{27}H_{43}O_4N$ as shown in Table II.

Peimisine Oxime, $C_{27}H_{44}O_4N_2$.—A mixture of 0.1 Gm. of peimisine, 0.1 Gm. of hydroxylamine hydrochloride, and 0.2 Gm. of potassium acetate is dissolved in 20 cc. of water in the presence of one drop of acetic acid and warmed on the water bath for about one hour. After standing overnight the clear aqueous solution is made alkaline with potassium carbonate and the precipitate extracted with a mixture of ether and chloroform. The ether-chloroform extract, when dried and distilled, leaves behind the required oxime which separates from acetone as a crystalline powder, m. p. 196°. Its hydrochloride crystallized from alcohol in prismatic

* Received Dec. 30, 1946, from the Institut de Materia Medica, National Academy of Peiping, Shanghai, China.
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TABLE II CHEMICAL COMPOSITION OF THE COMPOUNDS DESCRIBED IN THIS PAPER

Name	Formula	Percentage Composition															
		Carbon		Hydrogen		Nitrogen		Chlorine		Platinum or Gold	
		Calcd	Found	Calcd	Found	Calcd	Found	Calcd	Found	Calcd	Found
Peimisine	$C_{27}H_{44}O_4N$	72.75	72.78	9.73	9.81	3.14	3.26	3.09			
Peimisine Hydrochloride	$C_{27}H_{46}O_4N \cdot HCl$								7.38	7.13	7.38				
Peimisine Aurichloride	$C_{27}H_{46}O_4N \cdot HCl \cdot AuCl_3$											25.10	21.93	21.78
Peimisine Oxime	$C_{27}H_{44}O_4N_2$					6.08	6.07	6.20						
Peimisine Oxime Hydrochloride	$C_{27}H_{44}O_4N_2 \cdot HCl$								7.13	7.18	7.21			
Peimiphine	$C_{27}H_{46}O_4N$	74.93	74.75	9.93	10.72	9.99	10.71	3.23	3.36	3.17				
Peimiphine Hydrochloride	$C_{27}H_{46}O_4N \cdot HCl$								7.56	7.75	7.63			
Peimiphine Aurichloride	$C_{27}H_{46}O_4N \cdot HCl \cdot AuCl_3$											25.53	25.53	25.70
Peimidine	$C_{27}H_{45}O_2N$	78.00	77.93	78.01	10.92	11.24	11.37	3.37	3.20	3.07				
Peimidine Hydrochloride	$C_{27}H_{45}O_2N \cdot HCl$								7.85	7.85	7.61			
Peimidine Platinichloride	$(C_{27}H_{45}O_2N \cdot HCl)_2PtCl_4$											15.72	15.85	15.61
Peimitidine	$C_{27}H_{41}O_3N$	75.29	75.38	75.06	10.30	9.89	10.01	3.25	3.37	3.51				
Peimitidine Hydrochloride	$C_{27}H_{41}O_3N \cdot HCl$								7.59	7.68	7.76			
Peimitidine Aurichloride	$C_{27}H_{41}O_3N \cdot HCl \cdot AuCl_3$											25.59	25.60	25.64

needles, m. p. 256°. Analysis confirms its composition as indicated in Table II.

Peimiphine, $C_{27}H_{46}O_3N$.—After filtering off the insoluble peimisine hydrochloride, the alcohol-soluble part of the crystalline deposit obtained above is evaporated to dryness and converted into its free base again by dissolving in water, making alkaline with sodium carbonate, and extracting with ether. The ethereal solution is dried and distilled and the residue neutralized with hydrobromic acid in a little alcohol. On addition of a sufficient quantity of ether, peimiphine hydrobromide crystallizes out in rhombic prisms. Peimiphine is liberated from its hydrobromide by action of sodium carbonate and recrystallizes from a mixture of acetone and petroleum ether. It forms hard prisms, m. p. 127°, $[\alpha]^{21}_{D}$ −69. It is easily soluble in chloroform and alcohol, but much less so in ether. Its hydrochloride, prepared by neutralizing the pure alkaloid with hydrochloric acid in acetone, forms orthorhombic prisms, m. p. 287°, and is easily soluble in water or alcohol. Its aurichloride is obtained as a yellow amorphous powder in a similar way as peimisine aurichloride. The composition of peimiphine is determined to be $C_{27}H_{46}O_3N$ according to the analytical results given in Table II.

Peimidine, $C_{27}H_{45}O_2N$.—After the removal of peimisine and peimiphine hydrochlorides as a crystalline deposit as described above, the mother liquor is distilled to almost dryness and the residue taken up with water and filtered. The aqueous solution is made alkaline with sodium carbonate and the free base extracted with ether. The ethereal solution is dried and distilled and the basic residue neutralized with concentrated hydrobromic acid in a little alcohol. On addition of ether, peimidine hydrobromide crystallizes out in rhombic prisms, m. p. over 300°. It is soluble in cold water with difficulty. Peimidine is obtained from its hydrobromide by action of sodium carbonate in an aqueous solution and extraction with chloroform. When crystallized pure from 95% alcohol, peimidine forms orthorhombic prisms, m. p. 222°, $[\alpha]^{26}_{D}$ −74°. It is only sparingly soluble in ether or acetone but easily soluble in chloroform or alcohol. Its hydrochloride forms rhombic prisms, m. p. 318°, easily soluble in alcohol or water. Its platinichloride is obtained as an orange amorphous powder by precipitation in aqueous solution in the presence of one drop of hydrochloric acid. The analytical data as given in Table II indicate its formula to be $C_{27}H_{45}O_2N$.

Peimitidine, $C_{27}H_{41}O_3N$.—The mother liquor of peimidine hydrobromide as described above is evaporated to dryness and the residue taken up with water and filtered. The free base is regenerated from the aqueous solution with sodium carbonate and extracted with ether. The ethereal solution is dried and distilled and the residue neutralized with concentrated hydrochloric acid in acetone. On long standing, peimitidine hydrochloride crystallizes out in rhomboid prisms. When recrystallized repeat-

edly from a mixture of alcohol and acetone, it has a melting point of 291° with decomposition, easily soluble in absolute alcohol or water. Peimitidine is obtained from its pure hydrochloride in a usual way and crystallizes from a mixture of acetone and petroleum ether in hard prisms, m. p. 188°, $[\alpha]_D^{20}$ −68°. It is easily soluble in most organic solvents, but much less so in ether. Its platinichloride is very soluble in water, but its aurichloride is easily obtained as an amorphous powder by precipitation in an aqueous solution. It has the composition of $C_{27}H_{44}O_3N$ as shown in Table II.

SUMMARY

From the mother liquor of peimine and peiminine, two principal alkaloids isolated from the Chinese drug, Pei-Mu, identified as *Fritillaria Roylei*, there have been isolated four other minor alkaloids to which the names peimisine, peimiphine, peimidine, and peimitidine are respectively assigned. Their formulas, melting points, and specific rotations are given in Table I. They are present in the drug only to the extent of 0.001 to 0.002 per cent. Peimisine easily forms an oxime, m. p. 196°.

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The Comparative Chronic Toxicities of Fumaric, Tartaric, Oxalic, and Maleic Acids*

By O. GARTH FITZHUGH and ARTHUR A. NELSON

Data are given on the effects of the oral administration of fumaric, tartaric, oxalic, and maleic acids. The results of the study indicate a low degree of toxicity for fumaric, oxalic, and tartaric acids. The oxalic and tartaric acids were not toxic in concentrations as high as 1.2% and fumaric acid was toxic only at the 1.5% concentration. Maleic acid was toxic at concentrations of 0.5% or more.

THE SIMILAR laxative action produced by the salts of fumaric and tartaric acids suggested a comparative survey of the chronic toxicities of these acids. Because of a low acute toxicity of fumarates to animals and of their efficacy as compared with salts of tartaric acid, recent investigators (1–6) have proposed a substitution of fumarates for tartrates in laxative preparations. The nephropathic action of large acute doses of tartaric acid (7) does not appear to occur in animals given similar doses of fumaric acid (1); however, no lifetime study has been conducted with either of these acids. The presence of large amounts of oxalates in certain foods (8–12) and the isomerism of maleic and fumaric acids suggested the inclusion of oxalic and maleic acids in a two-year study of the chronic toxicities of these 4 dicarboxylic acids. The many experiments reported in the literature on the toxicity of oxalic acid have been either acute or short-term chronic experiments.

* Received Jan. 14, 1947, from the Division of Pharmacology, Food and Drug Administration, Federal Security Agency, Washington, D. C.

EXPERIMENTAL

Two experiments were conducted in which groups of weanling rats (twenty-one days) from our colony of Osborne–Mendel strain were started on diets containing one of the above-named dicarboxylic acids. A total of 420 rats was used in these experiments. In the first experiment 12 groups of 24 rats, equally divided between the sexes, were fed on diets containing 0.1, 0.5, 0.8, and 1.2% tartaric, fumaric, and oxalic acids, respectively. In a second experiment, in order to compare more closely the toxicities of fumaric and maleic acids, 6 groups of 12 male rats were fed on diets containing 0.5, 1.0, and 1.5% fumaric and maleic acids, respectively. There were 48 control animals for the first experiment and 12 con-

trols for the second experiment. Ground commercial rat biscuits with 1% added cod-liver oil served as the basic diet. The acids were mixed with the basic diet by means of a rotary batch mixer. All animals were kept in individual cages in a room with the temperature and humidity controlled for the duration of the experiment and were given free access to their respective diets and water. The weights of individual animals and their food consumption were determined at weekly intervals.

RESULTS

Since the two experiments were started at the same time and were part of the same study, they will be discussed together.

The Effect on Growth Rate and Food Consumption.—An inspection of Table I shows that all animals, with the exception of those on the two highest dosages of maleic acid, made a normal gain in weight during the first fifty-two weeks of the experiment. The slight differences between other groups of experimental animals and the controls were not significant ($p = 0.05$, or less, is significant). Individual growth curves of rats from the same litter and the composite growth curves of the several groups showed that there were some variations in the retarding effect of the acids both during the fast growing period and during the plateau period. Analyses, therefore, were made on the gains in weight for the first twelve weeks and for the first twenty-six weeks of the experiment. These analyses showed a significant difference between the mean gain in weight of the control animals and the mean gains in weight of the groups on 1% and 1.5% maleic acid, but showed no difference between the mean gain in weight of the controls and that of any group of fumaric, tartaric, or oxalic acids. Thus, of the 4 acids used in this study, only the maleic at concentrations of 1%, or more, retarded the growth rate of the rats.

When the data for the weekly food consumption of each group during the first twenty-six weeks and during the second twenty-six weeks of the experiment were analyzed, the differences between the control and experimental animals were not statistically significant.

The Effect on Mortality.—Statistical tests cannot be applied to the mean survival time since all surviving animals were sacrificed at the conclusion of two years on the experiment. Most of the deaths, except for a few animals distributed throughout all groups and killed because of middle ear disease, occurred during the second year of the experiment. The number of animals surviving at eighteen months and at two years, therefore, were selected as estimates of relative toxicity. At eighteen months the maleic acid at concentrations of 1% and 1.5% in the diet increased the mortality rate; however, the difference between the mortality rate of either group of experimental animals and that of the controls was not significant. At two years the maleic acid had increased the mortality rate significantly. All of the 12 experimental animals on 1.5% and 10 on each of the other concentrations of maleic acid died whereas 6 of the controls survived to the end of the experiment. Likewise at two years there were only 2 animals living on 1.5% fumaric acid; other dosages of fumaric acid had no effect on mortality rate. There was no significant difference between the mortality rate of the rats on any dosage level of tartaric, or oxalic acid and that of the controls.

TABLE I.—MEAN GAIN IN WEIGHT OF RATS FED DIETS CONTAINING FUMARIC, TARTARIC, OXALIC, OR MALEIC ACID FOR A YEAR

Substance	Dosage, %	No. of Animals	Sex	Mean Gain in Weight, Gm.	Standard Error of Mean, Gm.
Fumaric Acid	0.1	11	M	452.9	±19.0
	0.1	10	F	288.0	±10.0
	0.5	10	M	444.4	±20.7
	0.5	12	F	269.7	± 9.6
	0.8	9	M	429.6	±11.0
	0.8	12	F	265.2	± 5.7
	1.0	10	M	468.8	±25.9
	1.2	11	M	466.9	±14.6
	1.2	12	F	280.0	±10.2
	1.5	7	M	459.9	±23.2
Tartaric Acid	0.1	9	M	459.9	±16.7
	0.1	12	F	278.2	±10.6
	0.5	11	M	450.5	± 5.7
	0.5	10	F	286.3	± 6.1
	0.8	8	M	464.8	±16.7
	0.8	10	F	270.4	± 6.9
	1.2	9	M	437.8	±11.0
	1.2	11	F	258.0	± 7.5
Oxalic Acid	0.1	11	M	453.9	±19.1
	0.1	10	F	272.5	±11.1
	0.5	8	M	444.4	±20.7
	0.5	12	F	275.3	± 2.9
	0.8	10	M	464.2	±16.1
	0.8	11	F	259.8	± 3.4
	1.2	11	M	451.9	±15.6
	1.2	10	F	261.4	± 9.1
Maleic Acid	0.5	9	M	447.1	±15.5
	1.0	7	M	372.8	±20.6[a]
	1.5	9	M	274.3	±18.4[b]
Control		31	M	464.9	±13.4
		22	F	274.4	± 9.8

[a] $p < 0.05$.
[b] $p < 0.001$.

Pathology.—For reasons unconnected with these experiments a greater amount of microscopic pathological examination was done than would have been necessary to demonstrate the relatively minimal nature of the pathological changes produced by the acids. Because our nearly negative findings are based on a large amount of material, it is felt that they are worth presenting.

Autopsy was done on nearly all the rats used in these experiments, a few being omitted because of advanced post-mortem autolysis. As the animals were received in the pathology laboratory, alternate ones were taken for microscopic sectioning. In 213 rats, hematoxylin-eosin stained paraffin sections of

the following structures were routinely made: lung, heart, liver, spleen, pancreas, stomach, small intestine, kidney, adrenal, and testis. The following additional structures were sectioned frequently enough to make it reasonably certain that no changes in them were being caused by the acids: colon, bone marrow, leg bones, leg muscles, lymph nodes, uterus, ovary, thyroid, and parathyroid.

In all the foregoing structures, no difference could be seen between treated and control animals except the following. Slight periportal hypertrophy of the hepatic cells, coincident with slight centrolobular atrophy, was seen in 15 instances (11 in animals fed oxalic acid, 5 of these in the 0.1% group; none in controls). Enlarged and irregularly shaped epithelial cells in small to moderate numbers of renal tubules, generally the proximal convoluted, were seen in 4 rats on 1.5% and in 3 rats on 1.0% maleic acid, and in no other rats in the entire series. Rats fed 1.5% maleic acid had distinctly more atrophy of the liver and less focal calcification in large arteries than did any of the other groups, and, together with those receiving 1.5% fumaric acid, showed more atrophy of the testis than did any other group. Inanition is at least partly responsible for the atrophy of the liver and testis just mentioned. It may be of significance that 2 rats fed fumaric acid (1.0% and 0.5%, respectively) showed phlegmonous gastritis, a condition not seen in any other group.

With the few and relatively minor exceptions noted in the preceding paragraph, the gross and microscopic findings in this series of rats showed no difference between control and treated animals. Tumors and other more or less frequently occurring spontaneous diseases of older rats, such as periarteritis nodosa-like arterial lesions, bile duct proliferation in the liver, et cetera, showed no difference in incidence among the various animal groups.

SUMMARY

1. Toxic effects occurred in rats fed diets containing 0.5 per cent or more maleic acid and 1.5 per cent fumaric acid for two years. No significant toxic effect occurred in rats fed diets containing 1.2 per cent oxalic or tartaric acid.

2. Detailed microscopic pathological examination of 213 rats showed no major visceral damage from any of the acids at any level, and only relatively minor differences between control and treated animals.

3. Rats fed 1.5 per cent maleic acid showed more atrophy of the liver and (together with those on 1.5 per cent fumaric acid) of the testis than did the remaining groups. Inanition seems at least partly responsible for these differences.

4. Some of the rats fed oxalic acid showed slight periportal hypertrophy of the hepatic cells, and some of those fed maleic acid had atypical renal tubular ephithelial cells. Two rats fed fumaric acid had phlegmonous gastritis.

5. Maleic acid at a concentration of 1 per cent retarded the growth rate of rats.

6. All concentrations of maleic acid and the 1.5 per cent concentration of fumaric acid increased the mortality rate.

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The Separation of Penicillins by Partition Chromatography*

By HENRY FISCHBACH, THOMAS E. EBLE, and MERLIN MUNDELL†

Penicillin K was successfully determined in a mixture of penicillins by means of partition chromatography in which silicic acid is the adsorbent, a buffer solution the immobile solvent, chloroform and ether the mobile solvents. There was sufficient resolution of the other penicillins to permit the isolation and purification of a penicillin F.

EARLY in 1946, considerable concern was expressed by those employing penicillin therapy as to the penicillin K content of commercial penicillin. Accumulated evidence indicated that commercial penicillin produced since May, 1944, had been less effective against early syphilis than that produced prior to this date (1, 2). Further, it was known that proportionately greater amounts of penicillin K at the expense of penicillin G had appeared in the commercial product. At that time this laboratory issued a preliminary report (3) on a chromatographic procedure which had been found satisfactory for determining penicillin K in the presence of other known penicillins. The purpose of this paper is to present a comprehensive report of this laboratory's experience with partition chromatography in the field of penicillin assay and isolation.

Initially, an attempt was made to resolve the penicillins by the classic type of adsorption chromatography. Norite, combinations of norite and celite, talc, combinations of talc and celite, alumina, and acid washed alumina were tried as adsorbents. A number of solvents and combinations of solvents were utilized but at best a 75% recovery of adsorbed penicillins was effected with an acid washed alumina column and 80% with a combination of 2 parts norite to 3 parts celite (545). No resolution was perceived in the former and only a slight degree of differentiation in the latter. Since we used an iodometric procedure (4) to assay the fractions eluted from the columns, those solvents which contained impurities reactive with iodine were purified before use.

Considering the variation in the hydrophilic properties of the penicillins along with available information on the use of buffered silica gel columns, it was evident that partition chromatography might quantitatively resolve penicillin K from a mixture of penicillins. The initial silicic acid adsorbent was prepared by the Gordon, Martin, and Synge technique (5). Early in this work it appeared that a 20% phosphate buffer solution of *p*H 6.4 was desirable as the immobile solvent. Considerable time was spent in comparing the results obtained by use of the above activated type of silicic acid and those available on the commercial market. Only two commercial products failed to give the desired results The aqueous extract of one was strongly basic and the other was so fine that the percolation rate was impractical even with applied pressure. It was found that the commercial products could be used if the adsorptive capacity of the particular batch of silicic acid was first determined.

EXPERIMENTAL

Preparation and Utilization of Chromatographic Column.—In this laboratory small amounts of buffered solution are added to a weighed amount of the silicic acid and the mixture thoroughly macerated in a mortar after each addition. A point is reached where the silicic acid after grinding remains "sticky" and tends to "ball." Note is made of the per cent buffer required to bring about this degree of saturation. In preparing a column, 5% to 10% less buffer than that which resulted in the above saturation is used. The column is formed from a slurry of buffered silicic acid in chloroform which had previously been washed with water to free it of alcohol. The chloroform slurry of silicic acid in the column is subjected to an approximate pressure of 80 mm. for the purpose of packing down the adsorbent. This packing by pressure is continued until the volume of the

* Received Feb 13, 1947, from the Medical Division, Chemical Section, Food and Drug Administration, Federal Security Agency, Washington, D. C.

† The authors acknowledge the cooperation of the Division of [illegible] for the bioassays, and of [illegible] of Microbiology for the crystallographic data.

chloroform-silicic acid mixture remains relatively constant. Sufficient solvent is added as needed to keep the column from channeling and going dry. Care is taken to eliminate air pockets during the packing procedure, by rapidly rolling the column in a vertical position between the palms of the hands when necessary. For convenience the percolating rate used in this laboratory is adjusted to 25 ml./ten to fifteen minutes, although considerable latitude of the rate is permissible. Equally good results are obtained with percolating rates varying from 25 ml./seven minutes to 25 ml./thirty-five minutes. A 5-gallon carboy, equipped with manometer and rubber bulb, is used as a simple source of controlled air pressure.

There are a number of factors which make chloroform the mobile solvent of choice at this stage for placing the penicillins on the column. The free penicillins are extracted from the aqueous phase at *p*H 2 under which condition the partition coefficient of the penicillins between chloroform and water is very favorable. The low solubility of water in chloroform minimizes that amount of *p*H 2 buffer carried along in the organic phase. In addition, penicillin X remains in the aqueous phase thus eliminating one penicillin prior to chromatographic resolution. Lastly, chloroform affords a concentrated narrow band of activity at the top of the column, whereas the use of a solvent, such as ether, results in a broad band of activity even with a restricted volume, thus limiting the length of column available for the development of the chromatogram.

The aqueous mixture of penicillin salts is acidified to *p*H 2 at 0° and extracted with three successive portions of chloroform. The combined extracts are added to the prepared column. After the chloroform solution has passed into the adsorbent, a 25-ml. portion of chloroform is added. As the last of the chloroform passes into the adsorbent, the eluent is added and the penicillins resolved. Peroxide-free ether saturated with water is the most satisfactory eluent of those tried and, since the operations are conducted in a 10° room to minimize the destruction of penicillin, its volatility is not a serious drawback. By the above procedure, no activity is found in the eluates until ether begins to come off the column. Ordinarily, 25- to 50-ml. fractions are collected and assayed by the iodometric procedure for total penicillin. The chloroform or ether fractions are assayed directly without a previous extraction into an aqueous medium.

Data.—The preliminary studies were limited to commercial penicillin since some of the pure penicillins were not readily available. It was evident from the start that one band of activity traveled very rapidly through the column and was eluted within a relatively few 25-ml. fractions. Subsequent to this band there followed several fractions with no activity before appearance of another band of penicillin. From this point there was some overlapping of the fractions collected although distinct maxima and minima were evident. The determination of the subtilis-staphylococcus ratios and the crystallographic properties of the combined fractions of any one band established the sequence in which the penicillins were being eluted from the column. Penicillin K was the active component of the first band, followed by penicillin dihydro F, penicillin F, and penicillin G.

A small quantity of pure penicillin F was prepared by combining the ethereal fractions of the penicillin F band resulting from chromatographic resolution, removing the ether by reduced pressure in the presence of a buffer, extracting into chloroform at *p*H 2, re-extracting with sodium hydroxide to a *p*H of 7, lyophilizing and recrystallizing from acetone. Subtilis-staphylococcus ratios, crystallographic data, and infrared studies (6) indicate a penicillin F of very good quality.

When relatively pure penicillins K and dihydro F became available through the National Research Council, known amounts of penicillins K, dihydro F, F, and G were compounded and assayed as above. The known mixtures were prepared as follows: weighed quantities of the sodium salt of each penicillin were individually dissolved in ice water and extracted in chloroform at *p*H 2. Each chloroform extract was assayed separately and definite aliquots of each penicillin extract were mixed together. The mixture was added to the column and the subsequent recoveries were based on the assays of the original chloroform extracts.

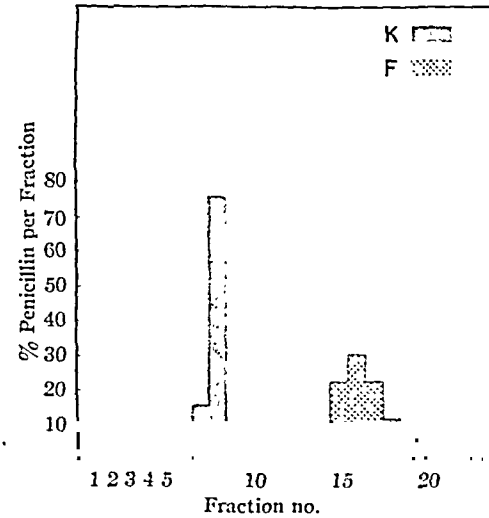

Figure 1

Figure 1 shows the separation of penicillins K and F on a column of Gordon, Martin, and Synge (5) activated silicic acid. In this study 101.3% of penicillin K and 101.6% penicillin F were recovered. Figure 2 represents the separation of penicillins K, F, dihydro F, and G on a column of commercial silicic acid. The recoveries in this case were 98.9% of penicillin K, 82.0% of penicillin dihydro F, 99.0%

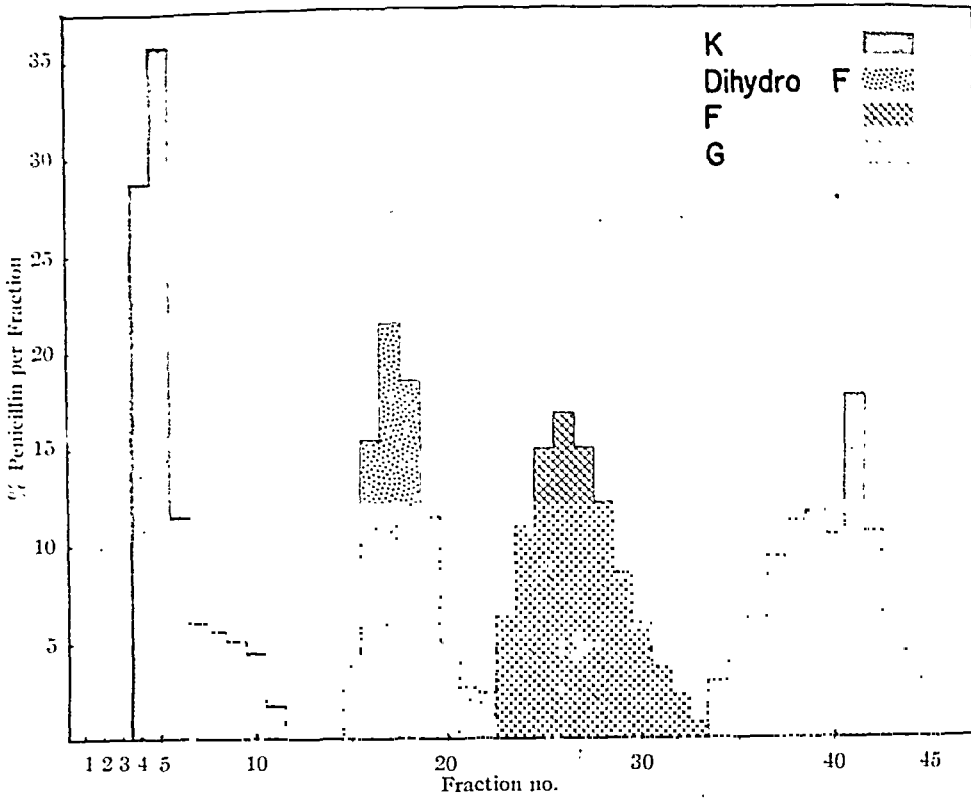

Figure 2

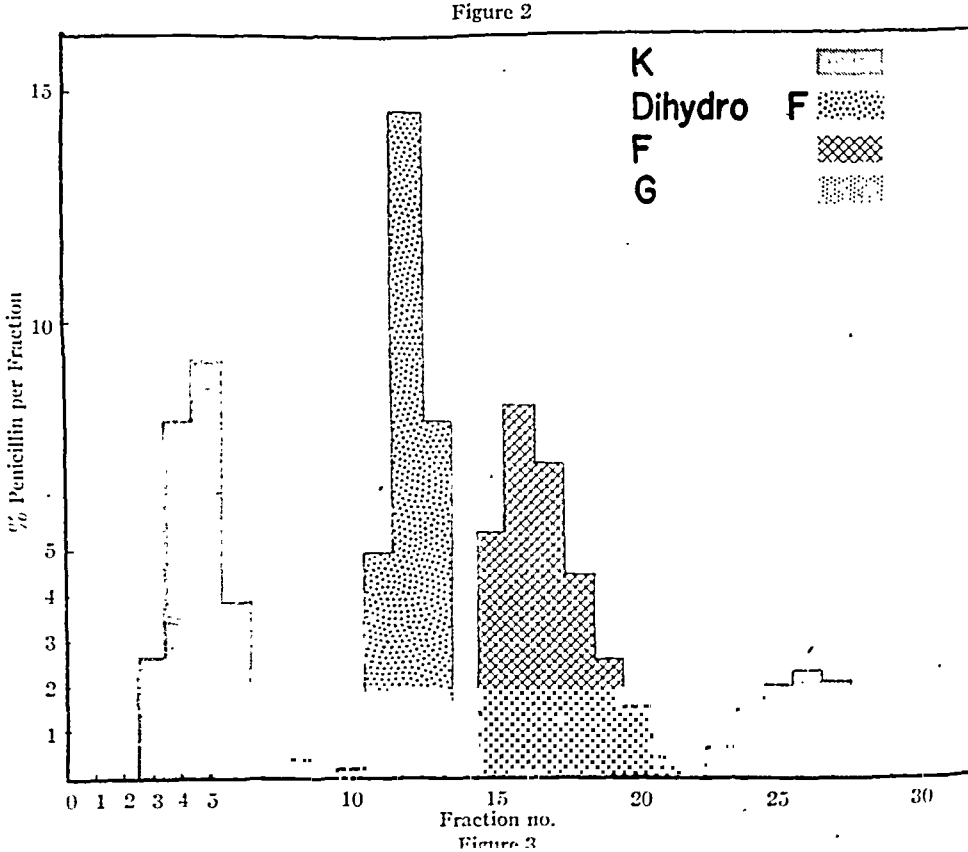

Figure 3

of penicillin F, and 98.3% of penicillin G. There is an unaccountable destruction of some of the penicillin dihydro F. Our results indicate that, at best, recoveries of between 80–85% of penicillin dihydro F can be obtained under the conditions necessary for optimum resolution of all the penicillins. Figure 3 shows the separation of a mixture of penicillins of unknown composition. This mixture was extracted directly into ether from a water solution at *p*H 2.0 and the extract placed on the column. The total recovery in this case was 94.0% of which 25.3% was penicillin K, 28.9% was penicillin dihydro F, 29.5% was penicillin F, and 10.3% was penicillin G. It can be inferred from other data that the inability to recover all of the penicillin was due to a partial destruction of penicillin dihydro F.

Since our primary interest had been the determination of penicillin K, it was advantageous that this penicillin should be the first to elute from the column and the one most completely separated under the above conditions. It was found that reproducible results, ±2%, for penicillin K were obtained under standardized conditions. This accuracy was obtained by arbitrarily fixing the dimensions of the columns, the pressure, the batch of silicic acid, the quantity of immobile solvent, and the quantities of mobile solvents. Having established the behavior of a mixture of penicillins under these conditions, it was possible to decide upon the quantity of eluate that would contain all of the penicillin K. Thus, only one fraction need be collected and assayed for penicillin K. In practice a subsequent fraction of 10 to 25 ml. was collected and assayed as a precautionary measure.

Previous experience with the iodometric assay for penicillin had been entirely on aqueous media. Consequently, the first assays entailed an extraction into water of each eluted fraction prior to the chemical determination. It was found, however, that the chloroform or ether fractions could be assayed directly by the iodometric procedure and the extraction step was eliminated.

SUMMARY

By means of partition chromatography, penicillin K was quantitatively determined in a mixture of penicillins. The same technique may be used for isolating and purifying the other penicillins. In this laboratory, a penicillin F of a high purity was so prepared.

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Book Review

Physical Constants of Hydrocarbons. Volume IV, Polynuclear Aromatic Hydrocarbons, by GUSTAV EGLOFF. Reinhold Publishing Corporation, New York, 1947. xi + 540 pp. 15 x 23 cm. Price $17.50.

For a review of Volume III of this series see THIS JOURNAL, **35**, 320(1946).

The source of most of the hydrocarbons described in this volume is coal tar and the commercial use is limited largely to dyestuffs. Many of these hydrocarbons, and more especially their derivatives, are of physiological interest. The carcinogenic hydrocarbons are in this group as are the hydrocarbon nuclei of the steroid compounds.

Like Volume III, this volume is a compilation of melting point, boiling point, and refractive index data of these polynuclear aromatic hydrocarbons. The same scheme of classification previously used is maintained throughout this volume. An additional advantage is the thorough documentation, several of the compounds having over fifty references cited.

In addition to the positive value of these data to the research workers, the gaps in the data focus attention on needs for further research.

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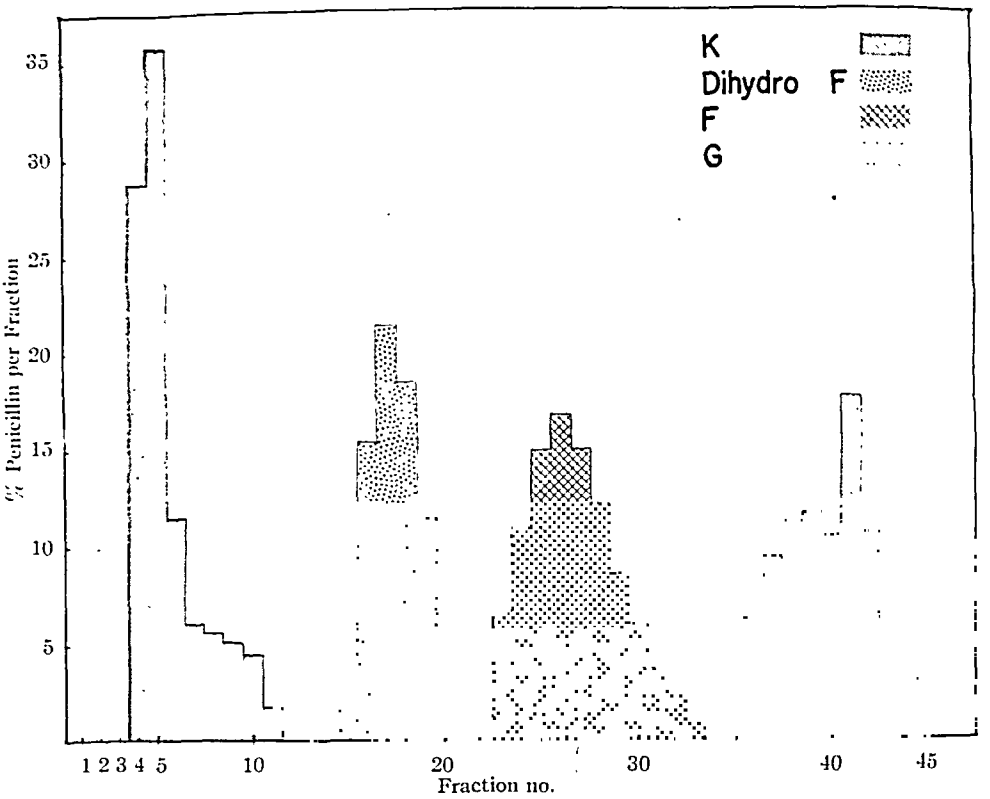
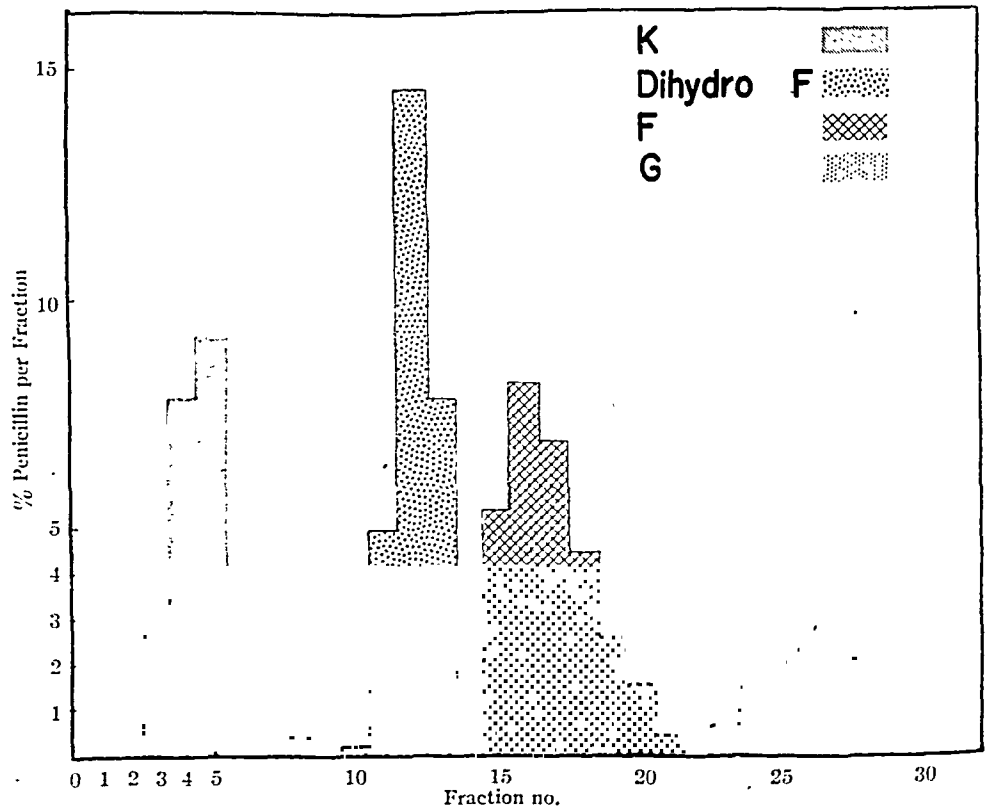


Figure 2



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Note

A Simple Color Reaction for Piperazine*

By ROBERT D. BARNARD

WHILE attempting to develop a gasometric method for the determination of uric acid, solutions of the latter in piperazine were treated with potassium ferricyanide[1] in the extraction chamber of a Van Slyke manometric apparatus (2). On shaking, a lilac color which reached an intensity peak in about five minutes and then faded, was noticed. Piperazine solution, itself, gave the same color on ferricyanide treatment provided metallic mercury was present. Since the reaction is not given by other diazines or by diketopiperazine, a color test for piperazine was developed on this basis, particularly when it was found that the color could be stabilized by buffering at *p*H 9.

Conduct of the Test.—To about 5 cc. of a solution suspected of containing piperazine which should contain more than one in ten thousand parts, add 10 drops of 5% $K_3Fe(CN)_6$ solution, or if the latter is found to be decolorized, the addition is continued, dropwise, until a lemon-yellow tint persists. A droplet of metallic mercury is now added, the tube stoppered and vigorously shaken so as to disperse the mercury.

In the presence of piperazine, a lilac color appears and fades after about ten minutes. If the solution to be tested has first been saturated with $NaHCO_3$ by addition of an excess of the solid substance, a rose-red color develops and persists indefinitely.

Interfering Substances and Conditions.—Substances reducing ferricyanide, e.g., uric acid, interfere; these are circumvented by addition of an excess over that required to oxidize such substances. Protein-containing fluids such as blood serum become turbid on treatment with the mercury; the pinkish color can, however, still be detected. The urine, particularly after piperazine administration, contains polyphenols which give a brownish or reddish color with the ferricyanide alone (catechol reaction). They may be removed from the test fluid by preliminary extraction of the acidified specimen with ether.

DISCUSSION

The test is rapid and specific and can be conducted with reagents common to any laboratory. It has been used in the attempt to demonstrate the presence of piperazine in the urine of human subjects after ingestion of 1 Gm. of the material. The results were negative which confirms the statement of Sollmann that piperazine is probably not excreted as such (3). In this connection an enhanced catechol reaction has been noticed in such urines, so it is possible that piperazine is excreted as a phenolic oxidation product.

The mechanism of the reaction is not clear. The ferricyanide is not reduced during its course, so the reaction product may be similar in structure to that of mercury fulminate and ferricyanide (4) whose color is almost identical. In the latter case, the mechanism can be surmised. Fulminate has a nitrile oxide structure (5) and the polar tenaille is sufficiently acute to permit penetration of the 4° coordination ring of the iron with polar linkage in the 3^2 orbit. Such compounds have been termed "heptacovalent" by Coryell and Stitt (6) but actually they are hexacoordinated unipolar covalent Piperazine is aresonant and stereochemically either of the imine nitrogens would constitute an acute polar tenaille which could enter such a covalent linkage. Whether this is the proper explanation of the colored compound, however, remains to be determined.

SUMMARY

A simple color test for the detection of piperazine, employing common laboratory reagents, is described. Urine specimens from normal subjects ingesting piperazine, were negative by this test.

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* Received Feb. 10, 1947, from the Laboratory Service, Halloran General Hospital, Staten Island, N. Y.

[1] To oxidize the uric acid to allantoin and recover the evolved carbon dioxide. The method failed as ferricyanide oxidation of uric acid does not yield allantoin directly as reported (1) No carbon dioxide is evolved except after rigorous alkai treatment. The primary oxidation product is probably uroxanic acid.

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The Effect of Saccharin Ingestion on Blood Coagulation and the *in Vitro* Anticoagulant Effect of Saccharin and of Ferriheme*

By ROBERT D. BARNARD†

Saccharin has been found to possess an antiprothrombic effect *in vitro,* but it is inert to thromboplastin and to thrombin. Lithium ferriheme shows an antithrombic and anticoagulant action. Saccharin administered orally had no effect on prothrombin time and showed no antagonism or synergism with dicoumarol.

A WEALTH of publications has arisen around possible toxicologic features of saccharin. Definitive conclusions are summarized in Sollman's (1) textbook. Though legislation in several states governs the distribution and sale of saccharin, about the only derogatory statement marshalled, and that a negative one, is that the substance is devoid of "food" value, precisely the feature of its greatest utility for thousands.

Within recent months, the absolute use of saccharin has increased enormously due to the relative absence of sucrose from the retail market. The numbers of temporary or occasional users must have multiplied. Frequently a chemical or pharmaceutic which while in restricted use is regarded as innocuous, is liable through extension of use to uncover instances of toxicity or sensitivity. No such report seems to have emanated from the recent increased usage of saccharin. The present study failed to show any profound effect of ingested saccharin on the blood coagulation mechanism.

This study was deemed desirable because of a hitherto unreported *in vitro* antiprothrombic effect of saccharin. During a trial of laboratory anticoagulants, Barnard and Rein (2) found that this substance would inhibit coagulation. Incubation of thromboplastin with saccharin did not inactivate the former and though calcium would not coagulate saccharinized blood or plasma, thrombin did so immediately.

Because theoretic interest attaches to new antiprothrombics, the clot inhibiting effect

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was subjected to detailed examination. In addition, the effect of saccharin ingestion on this phase of blood coagulation was studied.

EXPERIMENTAL

The Antiprothrombic Effect of Saccharin *in Vitro*.—Saccharin or its sodium salt (soluble saccharin) was added to freshly collected oxalated or citrated plasma for determination of antiprothrombic effect. In testing for direct anticoagulant effect, human blood samples were drawn by venipuncture directly into centrifuge tubes containing measured quantities of 25% aqueous sodium saccharin. Clotting time was determined by the Lee–White method (3). Prothrombin time was measured by a modified Quick (4) procedure to be described. Because of the low solubility of *o*-benzosulfinide (with consequent small antiprothrombic effect) it was necessary in some instances to use less than the standard quantity of thromboplastin in order to elicit appreciable gradations in degree of antiprothrombic effect observed. Thrombin time was measured by the method described by Quick (5).

About 100 mg. % of either the acid form or the sodium salt of saccharin are required for measurable anticoagulant effect and from 1.5% to 2.0% for complete coagulation inhibition. Unless the addition of saccharin is immediate, no anticoagulant effect is produced. Because of the technical difficulty of admixture immediately on its withdrawal and the fact that blood is partially hemolyzed by effective anticoagulant concentrations of saccharin, the latter is unsuitable as a general laboratory anticoagulant, though other sulfinides might profitably be explored in this connection.

Saccharin apparently effects the prothrombin-thrombin conversion. Using the regular "prothrombin time" measurement, this effect is not exhibited by the acid form of the material since the solubility of the latter is so limited. The sodium salt in 0.5% concentration is necessary to measurably affect this test whereas 1% of sodium saccharin will abolish coagulation completely. The observed effects of saccharin on the "prothrombin time" are confined between these limits. When the thromboplastin concentration is adjusted (as in some of the clinical procedures) so that a normal plasma shows an average time of twenty seconds, 5 mg. % will increase the time to twenty-four or twenty-five seconds. It was on this basis that an attempt was subsequently made to demonstrate an *in vivo* effect of saccharin on the coagulation mechanism.

The Antithrombic Effect of Lithium Ferriheme.—Saccharin combines with the ferriheme radicle of methemoglobin (6). An attempt was made to neutralize the antiprothrombic effect of saccharin with lithium ferriheme. This trial failed of its purpose; it uncovered instead an unanticipated antithrombic action on the part of this ferriheme.

When lithium ferriheme is added to plasma, the latter is rendered incoagulable by thrombin. No appreciable anticoagulant effect was elicited by 100 mg. % of lithium ferriheme added to freshly drawn blood. Twenty milligrams-per cent will delay the clotting of oxalated or citrated plasma, the thrombin time (Quick, 5) in this instance being 10 instead of the normal three seconds. Forty milligrams-per cent lengthens this to thirty-five seconds while 60 mg. % completely prevents the clotting of plasma by full strength thrombin. Addition of calcium salts and/or thromboplastin is likewise ineffective in clotting plasma containing lithium ferriheme. Lithium salts (citrate, carbonate, sulfate) have no effect on the clotting of plasma by thrombin. It is concluded that the antithrombic effect of lithium ferriheme resides in the iron porphyrin fraction which can combine with many hydracid anions including sulfhydryl. Aside from possible bearing on the various proposals of the sulfhydryl mechanism of blood coagulation (7), the results with lithium ferriheme are of interest since the growing field of the prophylaxis of thrombotic conditions necessitates continued search for diverse substances which inhibit clotting.

TABLE I.—THE EFFECT OF SACCHARIN ON THE PROTHROMBIN CONVERSION

Concentration of Saccharin in Plasma, (mg. %)	0	70	140	210	280	350[a]
Prothrombin Time, secs[b]	34.4	40.8	46.8	51.2	54.7	65.4
Prothrombin Time, secs[c]	14.8	17.2	18.6	20.2	21.2	22.6
Thrombin Time[d]	4	3	5	4	5	5
Thrombin Time[e]	6	6	7	7	6	7

[a] Saturated with saccharin at 37.5°.
[b] Half strength rabbit-brain thromboplastin—citrated plasma.
[c] Viper venom thromboplastin—oxalated plasma.
[d] Citrated plasma
[e] Oxalated plasma.

The Effect of Ingested Saccharin on the Blood Clotting Mechanism.—From the concentrations of saccharin found antiprothrombic *in vitro*, it was conceivable that comparable orders of magnitude in the circulating plasma might be reached on protracted or heavy oral dosage. Prothrombin time determinations made on the blood of 5 "routine" users showed no significant difference from the normal. No inordinately "heavy" users were available so that trial of theoretically sufficient oral dosage was made on ten subjects. This consisted of 500 mg. per day, by capsule, in 3 divided doses, each with a meal

The Experimental Series.—Four of the subjects

were cases of overt thrombophlebitis confirmed by clinical findings and phlebographic study. Two additional subjects had multiple (disseminated) sclerosis, in which the use of an antiprothrombic, dicoumarol, has been advocated. The other four subjects were doubtful cases of thrombophlebitis or phlebothrombosis, the diagnosis having been made without objective confirmation.

Two of the frank and two of the questionable thrombotic cases received dicoumarol in conjunction with saccharin. This was to determine possible synergism or antagonism of the two. All other patients. after a two-week course of oral saccharin alone, received a subsequent course of dicoumarol in order to rule out the possibility of refractoriness to antiprothrombics.

The Determination of Prothrombin Time.—All blood was collected one or two hours after the breakfast dose of saccharin. Citrate-Vacutainers (Becton–Dickinson Company) specially prepared to contain 0.2 cc. of 20% trisodium citrate, were used (8). This solution is somewhat viscous and coats the internal wall of the vacuum tube so that the first contact of blood after leaving the needle is with the concentrated anticoagulant solution. The vacuum aspiration draws the blood rapidly through the needle and rapid admixture with citrate is assured. Premature prothrombin-thrombin conversion is thus almost instantaneously inhibited. For even more perfect control in this connection, an expedient suggested by Whitby and Britton (9) has recently been adopted. The needle through which the blood is aspirated has been sterilized by immersion in boiling, light liquid petrolatum. The lumen of the needle retains a film of this "unwettable" material during the aspiration. This refinement was not used in the presently incorporated series.

In some independent studies, it has been found that ordinary collection by dry syringe or the use of dry oxalate may initiate premature thrombin formation. The natural antithrombins are thus neutralized before the test proper is conducted. This may lead to erroneous shortening of the prothrombin time. Theoretically, at least, it may be advisable to avoid particulate matter in the plasma before the actual determination is made and, for that reason, citrate anticoagulant has been adopted as our régular procedure. Prothrombin time determinations on citrated samples have nevertheless been found to be slightly shorter on the average than on oxalated plasma.

Prothrombin time on the citrated plasma, recovered by centrifugation or spontaneous sedimentation, was measured by the method of Quick (4) or that of Page and Russell (10). Normal prothrombin times for plasma collected in this manner and using Russell viper venom as thromboplastin range from ten to thirteen seconds.

RESULTS

No unequivocal effect of saccharin ingestion on blood prothrombin times was elicited. Saccharin could not be detected in the blood of such subjects at any time. (Saccharin when added to the plasma in minimal antiprothrombic concentrations can be identified by taste.) The 6 patients who were given saccharin, alone, for a period of two weeks and then placed on dicoumarol were all found to be responsive to the latter in the usual manner. One of the patients with questionable phlebothrombosis, after two weeks of oral saccharin followed by two weeks of dicoumarol therapy, was found, one month after discontinuance of all therapy to have a markedly sustained prothrombin time (fifty-five to sixty-five seconds) without hemorrhagic manifestation and correctable by Vitamin K administration. One patient with frank thrombophlebitis, on conjoint saccharin and dicoumarol, developed hematuria associated with hypoprothrombinemia three weeks after abatement of the venous condition and ten days after discontinuance of all therapy. For reasons cited below, these two instances are probably coincidental to the administration of the saccharin,

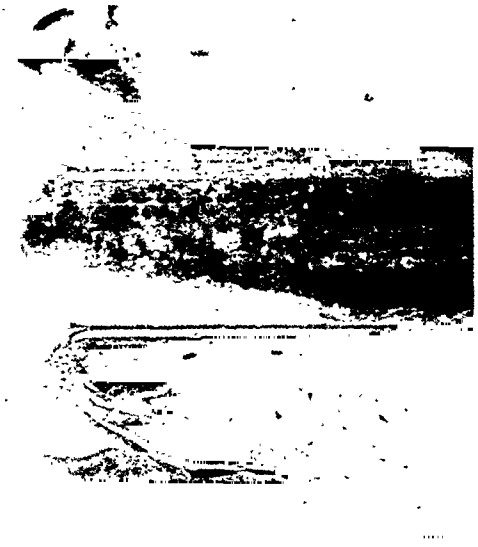

Fig. 1.—End Result of Prothrombin Time Determination Reaction Mixtures After Incubation

The clotted plasma of a patient receiving saccharin (upper) is compared with that of a patient on dicoumarol (middle) and a normal plasma (lower). All samples are of citrated plasma which have been clotted simultaneously with viper venom thromboplastin and calcium chloride solution and incubated for twelve hours at 37.5°. Until coagulation occurred during the conduct of the test, the reaction mixtures were almost water-clear. The tubes have been tilted to an almost horizontal position to demonstrate relative fluidity. Fibrinolysis affects exclusively the gelatinous material of the clot. The relative rapidity of this process in the clot from a subject receiving dicoumarol, at body temperature, suggests that the clot dissolution process may likewise be accelerated *in vivo* in the dicoumarolized patient.

the results from which can best be summarized as negative in all phases in so far as effect on the coagulation mechanism is concerned.

DISCUSSION

The results would be self-explanatory were it not for the fact that 2 of 10 patients receiving saccharin and dicoumarol, either concurrently or consecutively, showed an extension of antiprothrombic action not ordinarily ascribed to the latter drug alone This would seem to incriminate the saccharin However, perusal of case records of other patients receiving only dicoumarol, revealed this extension of seeming antiprothrombic action. Of 40 patients so medicated, extended and protracted hypoprothrombinemia was detected in three; it may have been present but undetected in a greater number. The mechanism of this protracted response to dicoumarol has not been investigated but on this basis it may be regarded as a possibly cumulative drug. With regard to the hematuria of a patient receiving saccharin and dicoumarol, two instances of hematuria have been observed after dicoumarol administration, alone.

Coagulated plasma of subjects receiving saccharin did not undergo more rapid *in vitro* fibrinolysis than that of control subjects but it is interesting that clots from dicoumarol subjects appeared to do so in some instances (Fig. 1). While not germane to the saccharin study, this observation deserves further exploration because of its clinical implications. The author has been under the impression for some time that the *usual* course of recanalization of a thrombosed vessel is through fibrinolysis of the contained clot rather than by the slower process of organization and capillary channelling and that this usual process is facilitated by the administration of dicoumarol. If this proves to be the case, it will mean that dicoumarol should be administered to assist in the dissolution of clots as well as for the ordinarily practiced prevention of clot accretion.

The absence of demonstrable anticoagulant effect from saccharin ingestion may be explained by its apparent absence in effective antiprothrombic concentrations from the blood after such ingestion. This was the case with ingested quantities far greater than would obtain in the ordinary situation. Whether the failure to reach a state of solution in the plasma is one of enteral absorption or of modification within the portal circulation is not known; in any event the dietary ingestion of saccharin would seem to be innocuous in so far as the production of hemorrhagic diathesis or predisposition is concerned.

CONCLUSION

The dietary use of saccharin cannot be interdicted solely on the basis of the demonstrated antiprothrombic effect which saccharin exerts *in vitro*. This conclusion is in line with common experience. Whether the same conclusion would be justified for the proposed use of saccharin, intravenously, in the determination of circulation time in human subjects can only be determined by further investigation.

SUMMARY

Saccharin has an *in vitro* antiprothrombic effect. It is inert to thromboplastin and to thrombin.

Lithium ferriheme, used in an attempt at the *in vitro* neutralization of the antiprothrombic effect of saccharin, proved to be antithrombic and anticoagulant.

Prothrombin time estimations present certain inherent difficulties which may be partially overcome by vacuum aspiration of the blood sample directly into concentrated citrate solution.

Ingestion of saccharin in amounts above those obtaining in ordinary dietary usage was found to be without effect on the prothrombin time. There is no synergism or antagonism between saccharin and dicoumarol. The latter itself may be a cumulative drug or one with protracted effect.

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A Pharmacological Study of Two Isomeric Sodium Hydroxymercuribenzoates*,†

By THOMAS J. HALEY‡, L. D. EDWARDS§, and C. H. JOHNSON‖

Several effects of two isomeric sodium hydroxymercuribenzoates have been determined and are reported in this paper. The effects of the two isomers have been found to be essentially identical.

THERE have been numerous investigations of the effects of mercurials on the cardiac tissue of various animals. Salant and Kleitman (1) showed that intravenous injections of large doses of a 1:5000 solution of mercuric benzoate, succinate, or acetate in dogs and cats caused cardiac depression and a fall in blood pressure. A further extension of this work (2) showed that the rate of intravenous injection had no effect on the production of the blood pressure fall, heart block, or the delirium cordis seen after total doses of 4 to 5 mg./Kg. of mercury as the succinate. In 1922, Salant (3) further stated that in dogs, cats, and rabbits mercurochrome produced the same cardiac effects as mercuric chloride, succinate, or acetate but larger doses were necessary. Isolated frog and turtle heart studies showed essentially the same toxic reactions as were seen with mammalian hearts. Jackson (4) confirmed these toxic effects on the dog heart using "Salyrgan" and "Novasurol." Salant and Nagler (5, 6) showed that mercuric chloride in concentrations of 1:50,000 and 1:10,000 caused a marked depression in the Straub frog heart preparation. The amplitude and strength of the beat were decreased and in thirty minutes the heart became feeble with the ventricle stopping before the auricle. Atropinization of the heart abolished the mercury effect caused by a dilution of 1:200,000 but mercury was not always effective in influencing the normal heart at this concentration. Macht (7) investigated the cardiac effects of mercuric chloride, mercuric benzoate, mercuric iodide in sodium iodide solution, mercurochrome, dimercury mercurochrome, merodicein, flumerin and mercurochrome plus mercuric chloride on the cat heart and concluded that inorganic and ionizable mercury compounds were more toxic than organic nonionizable mercury compounds. However, all mercury compounds produced the same cardiac effects. Debre, Leroux, and Hazard (8) showed that intravenous injection of the hydroxymercuripropanolamide of carboxyphenoxyacetic acid in a dose equivalent to 4 mg. of mercury per kilogram caused ventricular fibrillation and death. In 1940, Chastain and Mackie (9) showed that organic mercurials ("Esidrone," "Salyrgan," and "Novasurol") were not less toxic to the isolated turtle heart than inorganic mercurial (mercuric chloride and cyanide). Low concentrations caused auriculo-ventricular block while high ones caused sino-auricular block. Further, Johnson (10) tested "Salyrgan," "Mercuprin," mercuric and mercurous chlorides on the isolated turtle heart and concluded that these compounds were cardiac poisons because of their mercury content. Electrocardiographic studies by McCrea and Meek (11) indicate that the automatic and conducting mechanisms of the heart are attacked from above downwards. Similar studies by Masson (12) reported that there were pronounced alterations in the rate and amplitude of the heart beat under the influence of mercurials. Further, Salant and Brodman (13, 14), in studying the effect of mercurials on the atropinized cat heart, concluded that after mercury, larger doses of atropine were necessary for maintenance of the vagal terminal paralysis.

Mercury and mercurials have been shown to affect other tissues as well as those of the

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heart. Hinsdale (15) has shown that the action of mercury on the isolated cat intestine depended upon the concentration. Low concentrations caused a decreased height of contraction and high concentrations an immediate cessation of peristalsis with the tissue in an extreme state of contraction. This state was followed by a gradual relaxation and death. Salant and Brodman (16) showed that the soluble salts of mercury stimulated peristalsis in different animals. However, they did observe a primary depression followed by a secondary stimulation. Govorov (17) obtained similar results with the large intestine of the cat under the influence of "Novasurol." Dreyer (18) reported that the intestinal movements of the cat were increased by mercury salts as a result of a direct irritation of the intestinal mucosa.

Since both isomeric sodium hydroxymercuribenzoates contain a high percentage of mercury in their molecules and little is known about their pharmacological effects, this study was undertaken.

EXPERIMENTAL

Perfusion of the Frog Heart in Situ.—The frog heart was perfused by means of a Greene cannula using the method given by Sollmann and Hanzlik (19). The perfusion rate using Howell Frog Ringer's solution was about 1 cc. per minute. After the heart had established a constant rate and amplitude, a dose of the drug was added via the standpipe. In all cases the dosage of the drug was 2 cc. of a 1:5000 solution of either isomer. The dosage and strength were suggested for an oral preparation (20). After the heart had re-established a definite rate and amplitude of contraction from the first dose, a second dose was administered.

The kymograph records of ten experiments showed that 2 cc. of sodium *p*-hydroxymercuribenzoate solution caused a slowing of the heart rate on the average of from one beat per second to two beats every five seconds. At the same time there was better filling of the heart and a very slight increase in the amplitude of contraction as compared with the normal. After a second dose of 2 cc. there was an even greater decrease in the rate, averaging

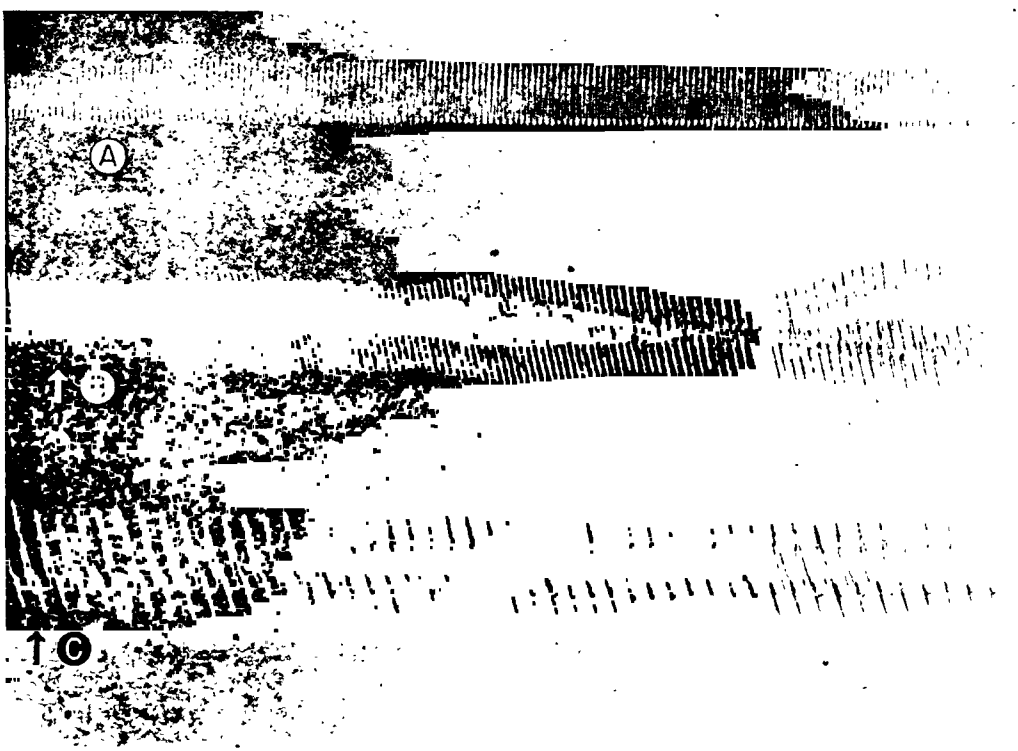

Fig. 1.—Record of frog heart perfusion *in situ*.

A, Normal Howell's Frog Heart Ringers Solution; *B*, 2-ml. Na-*p*-hydroxymercuribenzoate 1:5000; *C*, 2-ml Na-*p*-hydroxymercuribenzoate.

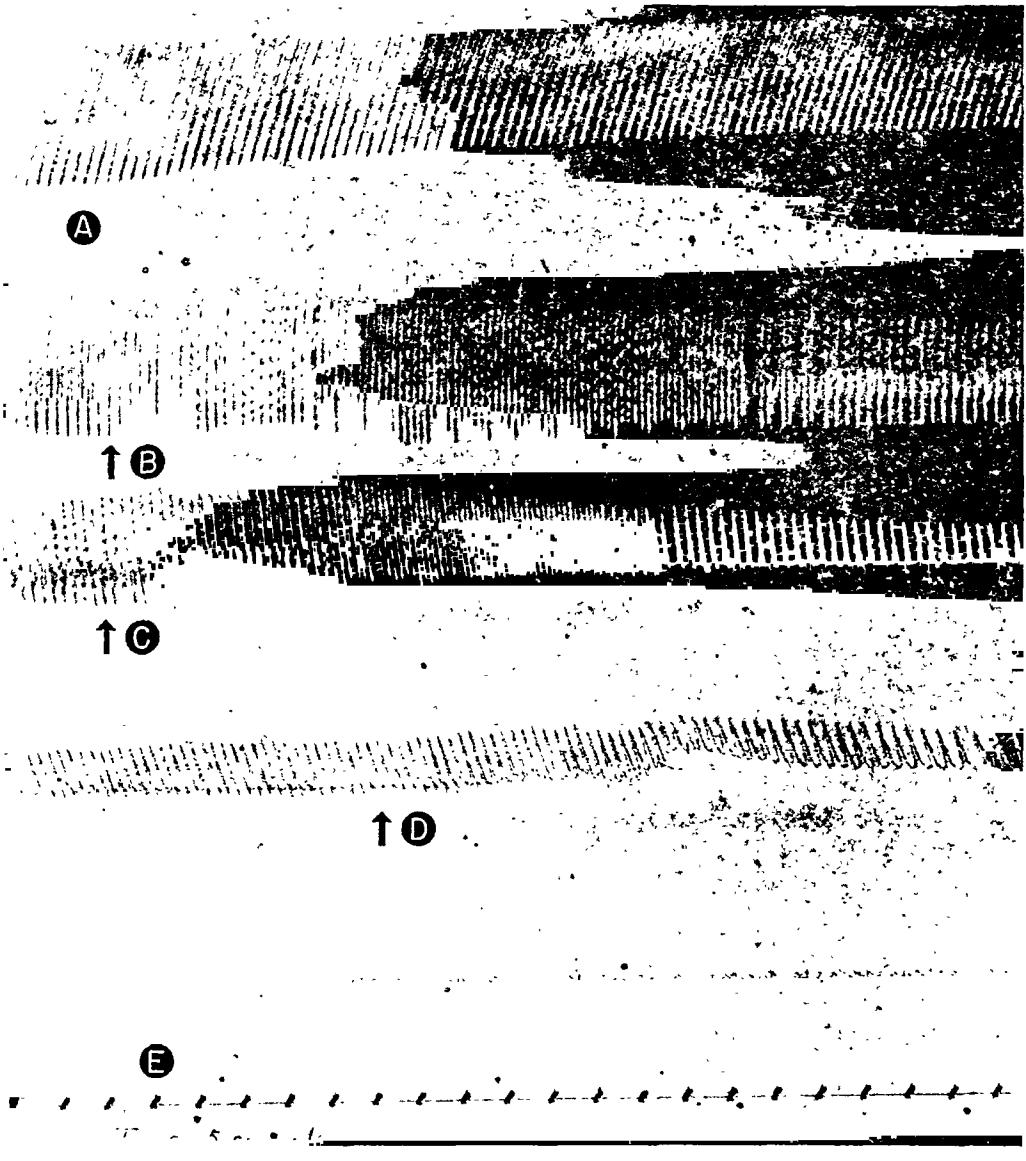

Fig. 2.—Record of frog heart perfusion *in situ*.

A, Normal Howell's Frog Heart Ringers Solution; *B*, 2-ml. Na-*o*-hydroxymercuribenzoate, 1:5000; *C*, 2-ml. Na-*o*-hydroxymercuribenzoate, 1:5000; *D*, 2-ml. Na-*o*-hydroxymercuribenzoate, 1:5000; *E*, Time 5 seconds.

one beat every five seconds, finally ending in a complete heart block. At the time of paralysis the heart was completely dilated. The records of the *ortho* isomer showed that a dose of 2 cc. caused a very slight temporary rise in the height of contraction followed by a decrease. The rate slowed very slightly, on the average from one beat per second to four beats every five seconds, at the same time there was better filling of the heart. After the second dose of 2 cc., there was a progressive decrease in the height of contraction until the auricle beat was no longer recorded. This was followed by a decrease in the rate, averaging three beats every ten seconds. The heart became completely dilated and complete heart block terminated these experiments. In several of the experiments both auricular and ventricular extrasystoles were noted but they were not prevalent and probably were due to pressure changes in the perfusion cannula. Figures 1 and 2 are typical results.

Perfusion of the Turtle Heart *in Situ*.—The results obtained with the frog hearts did not take into account the cardiac nerves, particularly the vagus. Another perfusion experiment using the turtle heart with an intact vagus was undertaken. The same method and conditions as with the frog heart were employed. After a normal record had been obtained, the vagus was stimulated electrically, then atropine sulfate solution 1:10,000 was painted on the heart to block all vagal action. When electrical stimulation showed the vagus to be blocked, a 2-cc. dose of the drug was given via the standpipe. From time to time the kymograph rate was increased to better analyze the heart beat.

With the *para* compound the first dose of 2 cc. caused very little change in the amplitude and rate of contraction. After a second dose of 2 cc. the amplitude was slightly decreased. Further, analysis of the reaction revealed that the auricles were beating four times to the ventricles once. With the third dose, this auricular reaction became more pronounced, with auricular beats from 14 to 32 for each ventricular beat. At this time the heart became dilated and the amplitude of contraction decreased to about one-fifth its original value. After a total dosage of 8 cc. the heart was completely dilated but not paralyzed, and the vagal blocking was still effective.

With the *ortho* compound the first dose of 2 cc. caused a very slight decrease in the amplitude of contraction followed by recovery. With the second dose the amplitude was decreased on the average to about half that of the normal. The third dose further reduced the amplitude of contraction with only the ventricle beat recording. Extrasystole were prominent. As the experiments progressed the rate of auricular beats to ventricular beats varied from 1:1 to 3:1 to 1:4 with no set order of reaction. The amplitude of contraction gradually decreased to about one-tenth its original height. After a total dosage of 10–12 cc. the heart was completely dilated but not paralyzed, and the vagal blocking was still effective.

When the heart could not be completely freed of blood, it was always able to withstand a much greater dosage of either of the drugs (24–30 cc.).

Effect on the Cat Blood Pressure and Respiration.—The procedure given by Jackson (21) was followed with one exception: the respiration was recorded by inserting a balloon into the thoracic region of the esophagus. The eighteen animals used, ten with the *para* and eight with the *ortho* isomer, were anesthetized with sodium pentobarbital, 35 mg. per kilogram body weight intraperitoneally. The drugs were administered via a cannula in the femoral vein. The first dose was 5 cc. of a 1:5000 solution, and this was followed at intervals of six to eight minutes with 10-cc. doses. A dose was always injected rapidly, requiring at the most only one and one-half minutes. Following each dose of the drug 3 cc. of saline were allowed to flow into the vein to wash all traces of the drug into the animal. The total amount of solution injected was 95 cc. equivalent to 10.564 mg. of mercury.

The *ortho* isomer caused a lowering of the cats' blood pressure of 20–30 mm. in the period of three hours. In several instances the rapid injection of the drug caused a shocklike state with the blood pressure falling to about 80 mm. from an initial value of 160 mm. However, the animal always recovered, and the pressure returned to approximately normal. The respiration was slightly increased in rate after each injection but there was an early return to its previous value. Other than this there was no outstanding effect on the respiration. In four animals defecation took place, probably because of reflex stimulation of the intestinal tract. Further, several animals urinated during the course of the experiments. In every case (six) where urination occurred the animal had a definite hemoglobinuria. This would indicate that the drug combined with and then hemolyzed the cats' erythrocytes.

The blood pressure and respiratory effects caused by the *para* compound were similar to those caused by the *ortho* compound. In five experiments, defecation was seen and five different cats showed hemoglobinuria.

Effect on Rabbit Erythrocytes *in Vitro*.—As hemolysis took place *in vivo* producing hemoglobinuria a similar result was expected to be observed *in vitro*. Five cubic centimeters of a 1:5000 solution of each of the isomers in normal saline hemolyzed 3 drops of rabbit blood in two and one-half hours. Crenation preceding this hemolytic action was observed under the microscope.

Effect on the Isolated Rabbit Intestine *in Vitro*.—The effect of these two salts on the excised rabbit intestine was studied by the Magnus method of recording the longitudinal contractions. The rabbit was anesthetized with 40 mg. of sodium pentobarbital per kilogram body weight intravenously. The animal was attached to a board and an incision was made on the ventral side from the sternum to the groin. Three centimeters of the small intestine were removed and at once attached to the apparatus. After allowing time for recovery, contractions were recorded on a slow drum. The mercurial compounds in 1:5000 aqueous solution were added directly to the bath in 1-, 2- and 5-cc. portions. With the 1-cc. dose there was little or no effect with either isomer. The second dose (2 cc.) of the *ortho* isomer caused an increase in tone, a slight decrease in amplitude, but no effect on the rate of contraction. After this dose the segment rapidly returned to normal but when the 5-cc. dose was added to the bath it caused depression with the segment barely contracting.

Results with the *para* isomer were similar but depression was seen even with the 2-cc. dose where there was a slight decrease in the amplitude of contraction but no decrease in rate. With the 5-cc. dose the effect seen was the same as with the *ortho* isomer.

Effect on the Rabbit Intestinal Loop *in Situ*.—At

the time of the intestinal segment experiments, three loops of small intestine with blood and nerve supply intact, 3 cm. long, were tied off. Into separate loops the following solutions were injected: 2 cc. of the *ortho* isomer, 2 cc. of the *para* isomer, and 2 cc. of normal saline. These loops were left in the closed abdominal cavity for three hours and then excised, washed, and examined with a hand lens.

The results of irritation caused by the solutions remaining in contact with the intestinal mucosa for three hours were:

Ortho isomer:—Area badly erroded, the membrane had a puffed and cooked appearance; in some cases the villi were erroded and absent but as a rule the villi were still prominent; one case of metallic mercury precipitation in the mucosa was seen.

Para isomer:—Area badly erroded, villi absent, membrane had a whitish cooked appearance with general capillary hemorrhage; some cases showed ulceration.

The fluid contained in the isolated loops was usually very viscous regardless of the isomer used. In all of the ten animals used the saline control loop remained normal.

plate 15.5 × 5 × 1 cm., through it were bored twelve holes 1.5 cm. in diameter and four holes 0.3 cm. in diameter. The latter were at opposite ends of the plate and were used to secure the instrument in place on the 25 × 8 cm. plywood baseboard. The twelve holes were lined with glass tubing of sufficient height to enable each of them to hold 2 cc. of fluid. Both the glass tubes and the plate were ground to make a level bottom.

When the instrument had been adjusted, the fluids of varying strengths were placed in the wells.

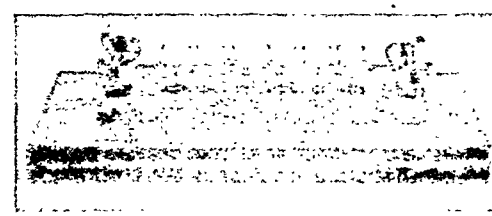

Fig. 3.—Instrument for the determination of peritoneal irritation.

TABLE I.—RESULTS OF PERITONEAL IRRITATION PRODUCED IN HALF AN HOUR BY *ortho* AND *para* SODIUM HYDROXYMERCURIBENZOATES

Well	Substances	Strength	Remarks
1	Saline control	0.85%	Tissue normal
2	*Ortho* isomer	1:5000	Tissue whitish[a]
3	Mercuric chloride	1:1000	Tissue extremely whitish and cooked
4	Mercuric chloride	1:10,000	Tissue whitish[a]
5	Mercuric chloride	1:50,000	Tissue whitish in streaks[a]
6	Mercuric chloride	1:100,000	Tissue slightly whitish
7	Saline control	0.85%	Tissue normal
8	*Para* isomer	1:5000	Tissue whitish[a]
9	Mercuric chloride	1:1000	Tissue extremely whitish and cooked
10	Mercuric chloride	1:10,000	Tissue whitish[a]
11	Mercuric chloride	1:50,000	Tissue whitish in streaks[a]
12	Mercuric chloride	1:100,000	Tissue slightly whitish

[a] Results appeared to be approximately equal.

Effect on the Rabbit Peritoneum.—Mercury compounds are all highly irritating to serous as well as mucous membranes. In order to determine the degree of irritation of both isomeric compounds on a serous membrane a special experiment was devised. This method utilizes the rabbit peritoneal membrane and the results obtained on this membrane should approximate those that would be obtained on a mucous membrane such as the oral mucosa.

The same animals used in the previous experiments were used for these tests. Two incisions, one at each end, were made on the right side perpendicular to the first longitudinal incision. All vessels cut by this procedure were clamped. The flap thus made was attached to a special board and secured to prevent the loose membrane from contracting. A twelve-welled instrument (Fig. 1) was then placed over the peritoneum and subjected to pressure just sufficient to prevent leakage.

This instrument was constructed from a brass

The observations were made after half an hour of contact with the tissue. The results of ten experiments are given in Table I.

DISCUSSION

The effects of the two isomers on the frog heart are similar to those reported for other mercurials by Salant (3) and Salant and Nagler (5, 6), but the effects on the atropinized turtle heart differed from those of the same investigators. This difference may be due to the difference in dosage used or it could be due to the fact that they used an ionizable mercurial whereas both the isomers investigated in this study had the mercury bound to the nucleus of the molecule. If the cats

used in this investigation had been atropinized a direct comparison could have been made with the other results reported by Salant and Brodman (13, 14) but as this was not done no statement can be made which would apply to the atropinized mammalian heart. In their effects on the blood pressure, respiration, intestinal contractions, irritant action, and erythrocytes the two isomeric sodium hydroxybenzoates studied agreed with the results reported for other mercurials.

SUMMARY

1. Both isomers show a similar depressant action on the perfused frog heart *in situ*.

2. No escape from atropine paralysis of the vagus was observed with either isomer on perfusion of the turtle heart *in situ*.

3. Neither isomer completely paralyzed the turtle heart, but auricular and ventricular extrasystoles were observed in all cases.

4. Neither the blood pressure nor the respiration are affected to any great degree when the isomers are administered intravenously. However, the stimulation of the defecation reflex indicated that the drugs had an irritant effect on the intact intestinal tract. Further, the production of hemoglobinuria demonstrates a hemolytic action by these compounds.

5. Both isomers hemolyzed rabbit erythrocytes *in vitro*. Microscopic studies showed that crenation preceded hemolysis, and that the solvent action of these compounds on the cell membrane took place slowly.

6. Both isomers showed an over-all depressant action on the isolated rabbit intestine.

7. Both isomers caused errosion of the intestinal mucosa and results indicated that prolonged contact between solutions (1:5000) of either of these compounds was very detrimental to mucous membranes.

8. An apparatus was devised which enabled an approximation of the irritant action of solutions of mercurials on serous membranes.

9. Both isomers showed an irritant action on the rabbit peritoneum which appeared equivalent to that obtained with 1:10,000 and 1:50,000 dilutions of mercuric chloride.

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The Assay of Aspirin, Acetophenetidin, and Caffeine Capsules*

By NANCY GREEN and MELVIN W. GREEN

Acetophenetidin is determined from the ethoxy content using a modified semimicro Zeisel apparatus. The ethyl iodide formed is collected in a bromine-potassium acetate-acetic acid mixture whereby iodate is formed and ultimately determined with thiosulfate. The aspirin is determined by extraction with sodium bicarbonate and subsequent bromination with Koppeschaar's reagent. After weighing the total chloroform extractive containing acetophenetidin and caffeine, the caffeine is determined by difference.

UPON THE admission of Aspirin, Acetophenetidin, and Caffeine Capsules to N. F. VIII, attention was turned to existing methods for the separation and determination of the components of the mixture. The method of the Association of Official Agricultural Chemists (1) (Method A) was the accepted method first used in this laboratory. This method, modified to some degree, was found to contain many possibilities for error, and gave varying results when applied to known mixtures and to commercial preparations of the capsules. When the Holt method (2) (Method B), based on an ether separation of the acetophenetidin and aspirin from the caffeine, was developed in an effort to decrease the time required for the assay, it was applied in this laboratory to known mixtures and to commercial samples, with somewhat variable results.

Recently a collaborative study (3) of the A. O. A. C. and of the Holt methods was conducted by members of the National Formulary Advisory Committee of the American Pharmaceutical Manufacturers Association and the American Drug Manufacturers Association. Erratic results were obtained and a majority of those engaged in the study could not recommend that either method be made official at this time. A method for the assay of these capsules in the British Pharmaceutical Codex is described and commented upon by Garratt (4), but its similarity to the A. O. A. C. method indicates that it would give similar results. Hilty and Wilson (5) have collected and simplified the directions for the A. O. A. C. methods of assay for Aspirin, Acetophenetidin, and Caffeine Capsules and similar preparations in a very useful paper.

The value and ease of alkoxy determinations in the assay of methyl cellulose pointed to the possibility of determining acetophenetidin by a similar method. This method (6) (Method C), which, for acetophenetidin, is semimicro, was employed on known mixtures and on commercial samples. The aspirin and caffeine were determined on a separate sample, separating the aspirin with bicarbonate and assaying it with bromine. The total chloroform residue (representing acetophenetidin plus caffeine) was then weighed, and the caffeine determined by difference, using the acetophenetidin figure determined separately by the ethoxy method.

A study of the three methods and some of their limitations has been made in this laboratory.

EXPERIMENTAL

Method A (Modified A. O. A. C. (1) Method)

Aspirin.—Weigh accurately the contents of twenty of the capsules and macerate a portion of the powder representing about 0.06 Gm. of caffeine with 25 cc. of chloroform for ten minutes. Filter through a small filter previously moistened with chloroform into a separatory funnel. Wash the filter with five 5-cc. portions of chloroform.

Extract the chloroform solution with two 50-cc. portions of 4% sodium bicarbonate solution and then wash with 20 cc. of distilled water. Combine the sodium bicarbonate solutions and wash water and extract with two 25-cc. portions of chloroform, collecting these chloroformic extracts in the separator with the original chloroform. Retain the chloroform solution for the acetophenetidin and the caffeine assays.

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Transfer the sodium bicarbonate solution into an Erlenmeyer flask and boil for five minutes. Add 5 cc of 20% sodium hydroxide solution and continue to boil for fifteen minutes. Cool and dilute to exactly 500 cc. with distilled water. Transfer a portion equivalent to approximately 0.065 Gm. of aspirin to an iodine flask. Add 50 cc. of 0.1 *N* bromine and 6 cc. of hydrochloric acid and set the flask aside for thirty minutes, shaking occasionally. Add 15 cc of potassium iodide T. S. and titrate the liberated iodine with 0.1 *N* sodium thiosulfate. Each cc of 0.1 *N* bromine is equivalent to 0.003 Gm. of aspirin

Acetophenetidin and Caffeine.—Evaporate the chloroform solution retained above, containing the acetophenetidin and caffeine, to a volume of about 10 cc. Add 20 cc. of 1% sulfuric acid solution, add a stirring rod, and heat on a water bath until all of the chloroform has evaporated, stirring occasionally Cool and decant through a tared Gooch crucible, previously dried to constant weight at 100°. Collect the filtrate in a 150-cc. beaker, retaining as much of the acetophenetidin as possible in the original beaker. Rinse the sides of the beaker containing the acetophenetidin with 5 to 10 cc. of chloroform, add 20 cc. of 1% sulfuric acid solution, and again heat on the water bath until all of the chloroform has evaporated. Cool, and decant through the above Gooch crucible. Wash the acetophenetidin quantitatively into the Gooch crucible with water, and wash the beaker and crucible with water until the filtrate measures approximately 75 cc. Dry the crucible to constant weight at 100° and weigh as acetophenetidin.

To the filtrate (containing the caffeine and a small amount of acetophenetidin) add 8 cc. of diluted sulfuric acid and evaporate on a steam bath to a volume of about 10 cc. Suspend the beaker in the steam bath and evaporate to a volume of 5 cc., add 10 cc. of water, and again evaporate, continuing the diluting and evaporating until the odor of acetic acid can no longer be detected in the vapors. Cool and transfer to a separator with a minimum amount of water, so that the final volume does not exceed 20 cc. Extract with three 50-cc. portions of chloroform, filtering each portion through a dry filter into a tared beaker. Allow the chloroform to evaporate with gentle heat, dry to constant weight at 80°, and weigh as anhydrous caffeine.

Wash the above filter used to dry the chloroform with 5 cc. of water and add the washings to the separator containing the hydrolyzed acetophenetidin. Add successive small portions of solid sodium bicarbonate until the solution is neutral, and then add a small excess. Add 50 cc. of chloroform and 10 drops of acetic anhydride, and shake vigorously. Allow the solutions to separate and withdraw the chloroform into a second separator containing 5 cc. of water. After washing the chloroform, pass it through a small dry filter into a tared beaker. Repeat with two additional 50-cc. portions of chloroform, washing each portion through the 5 cc. of water. Evaporate the solution to dryness on a steam bath. Add 1 cc. of chloroform and 1 drop of alcohol and again evaporate to dryness. Dry to constant weight at 100° and weigh as acetophenetidin, adding the weight to that of acetophenetidin obtained in the Gooch crucible.

Both the direct weighing of the separated aspirin and its determination by double titration, as in the A. O. A. C. method, were tried but abandoned in favor of the simpler bromination procedure.

It is of interest to compare our results (Table I) with those of a collaborative assay reported by Grove (7). In one sample, assayed by 5 laboratories, recovery of 96.9% to 99.4% aspirin was effected. Acetophenetidin was recovered in yields of 99.6% to 103.5%, while caffeine was 98.1% to 106.9% recovered. Both our results and those reported by Grove indicate that the method leaves much to be desired.

TABLE I.—RECOVERY OF ASPIRIN, ACETOPHENETIDIN, AND CAFFEINE FROM KNOWN MIXTURES AND COMMERCIAL CAPSULES USING METHOD A

Preparation		Recovery of— Aspirin, %	Aceto., %	Caffeine, %
Known Mixture		106.9	94.12[a]	106.5[a]
		105.1	92.00[a]	98.15[a]
Capsule A		100.7	89.78	84.78
		100.4	93.97	87.50
Capsule B	(1)	100.3[b]	95.32	82.34
		93.80	93.40	88.10
	(2)	97.50	90.84	86.00
Capsule C	(1)	97.97[b]	...	...
		99.10	95.06	73.82
	(2)	100.05	99.76	82.66
		...	98.56	80.52

[a] Determinations made on a mixture of acetophenetidin and caffeine only.
[b] Two separate samples of 20 capsules each were assayed

Method B (Modified Holt (2) Method)

Acetophenetidin.—Accurately weigh a sample of the mixture containing about 0.3 Gm. of acetophenetidin and transfer to a separatory funnel. Add 25 cc. of 10% hydrochloric acid and extract with water-washed ether, using one 40-cc. portion and five 20-cc. portions. Combine the ether extracts in another separatory funnel and wash with one 10-cc. portion of the dilute hydrochloric acid and one 10-cc. portion of water, washing these in turn through two 25-cc. portions of ether and adding to the original acid solution. (*Retain the acid solution for the caffeine determination.*) Combine the ether solutions and extract the aspirin with one 30-cc. portion of 5% sodium bicarbonate, one 5-cc. portion of bicarbonate, and two 10-cc. portions of water, washing each portion through two 25-cc. portions of ether and combining in another separator. (*Retain the aqueous solutions for the aspirin assay.*) Filter all ether extracts through a cotton pledget into a tared flask. Evaporate the ether, dry at 100°, cool, and weigh as acetophenetidin.

Caffeine.—Extract the original acid solution with four 30-cc. portions of chloroform, washing each in turn through 5 cc. of 2% sodium hydroxide and 5 cc. of water. Filter the chloroform extracts through a cotton pledget into a tared flask. Evaporate the chloroform, dry at 80°, and weigh as anhydrous caffeine.

Aspirin.—Transfer the sodium bicarbonate solution to an Erlenmeyer flask and boil for five minutes. Add 5 cc. of 20% sodium hydroxide and boil for fifteen minutes. Cool and dilute to exactly 500 cc. with distilled water. Transfer a portion equivalent to approximately 0.065 Gm. of aspirin to an iodine flask. Add 50 cc. of 0.1 *N* bromine and 6 cc. of concentrated hydrochloric acid and set the flask aside for thirty minutes, shaking occasionally. Add 15 cc. of potassium iodide T. S. and titrate the liberated iodine with 0.1 *N* sodium thiosulfate. Each cubic centimeter of 0.1 *N* bromine is equivalent to 0.003 Gm. of aspirin.

It was found advantageous to increase the amount of sodium bicarbonate solution used to extract the aspirin from 25 to 30 cc., and to use an additional extraction with 5 cc. of bicarbonate. All ether used for this method was previously washed with water, since U. S. P. ether contains alcohol, which may cause low aspirin and caffeine results, consequently making the acetophenetidin results high. The results are shown in Table II.

TABLE II.—RECOVERY OF ASPIRIN, ACETOPHENETIDIN, AND CAFFEINE FROM KNOWN MIXTURES AND COMMERCIAL CAPSULES USING METHOD B

Preparation	Recovery of Aspirin, %	Recovery of Aceto., %	Recovery of Caffeine, %
Known Mixture	100.90	91.64	91.74
	100.80	96.12	...
	98.93	97.47	102.80
	91.44	108.10	102.00
Capsule A	99.55	92.29	100.80[a]
	100.80	94.10	99.85
	96.98	97.92	89.85

[a] Determinations conducted on 3 separate samples of 20 capsules each.

Method C

Method C consists of a separate and accurate determination of acetophenetidin, a separation and assay of aspirin similar to that of Method A, and a subsequent determination of caffeine by difference from a total acetophenetidin and caffeine determination.

The acetophenetidin is assayed by an ethoxy determination in a modified Zeisel apparatus (6). This determination is based on the formation of ethyl iodide, which is swept over into the receiving vessel by a flow of carbon dioxide. The ethyl iodide is collected in bromine-potassium acetate-acetic acid solution and oxidized to iodate. The excess bromine is discharged with formic acid, and the iodate determined iodometrically, using potassium iodide and sodium thiosulfate solution. Samsel and McHard (6) obtained 24.9% ethoxy content from U. S. P. acetophenetidin, equivalent to 99.00% recovery of the sample.

In calculating the caffeine recovery, it is necessary to calculate from the results of the ethoxy determination the amount of acetophenetidin in the chloroform-soluble residue. This amount of acetophenetidin is subtracted from the total chloroform-soluble residue to obtain the amount of caffeine present.

Acetophenetidin.—*Reagents:* 1. Potassium acetate-acetic acid solution. Dissolve 100 Gm. of anhydrous potassium acetate in 1000 cc. of a solution containing 900 cc. of glacial acetic acid and 100 cc. of acetic anhydride.

2. Bromine-potassium acetate-acetic acid solution. Dissolve 5 cc. of bromine in 145 cc. of the potassium acetate-acetic acid solution. Prepare this solution fresh daily.

3. Sodium acetate solution. Dissolve 250 Gm. of anhydrous sodium acetate in 1000 cc. of distilled water.

4. Hydriodic acid. Prepare a constant-boiling mixture, b. p. 126°, by redistilling hydriodic acid over red phosphorus, while bubbling carbon dioxide through the distilling mixture.

5. Aqueous suspension of red phosphorus. Add about 30 mg. of red phosphorus to 50 cc. of distilled water.

Apparatus.—The details of the modified Zeisel apparatus are shown in Fig. 1.[1] The apparatus consists of a boiling flask, A, fitted with a side arm for the introduction of carbon dioxide and connected to column B, which serves to separate aqueous hydriodic acid from the volatile alkyl iodide, which then passes through an aqueous suspension of red phosphorus in scrubber trap C, and is absorbed in the bromine-potassium acetate-acetic acid solution in absorbing tube D. A thistle tube is attached above D for the introduction of the bromine solution.

Since it was found to be advantageous to use as few joints as possible, only one, a ground-glass joint, between A and B, is used in the apparatus as proposed by Samsel and McHard (6).

The carbon dioxide is passed from the cylinder through two empty bottles, used as surge chambers, to a rubber outlet tube equipped with a capillary tube containing a cotton wadding filter. This outlet tube is attached to the side arm of A.

As an oil bath, a dish filled with sufficient oil to allow immersion of the flask in the oil approximately to the level of the hydriodic acid is used. This bath is heated by means of an electric heater thermostatically controlled.

Procedure.—Fill the trap by pouring a small amount of the aqueous suspension of red phosphorus through the cup above D. Follow with a water rinse, using sufficient liquid to fill the trap about half full.

[1] We are indebted to the Dow Chemical Company for supplying us with this apparatus, a modification of which was reported by them (6).

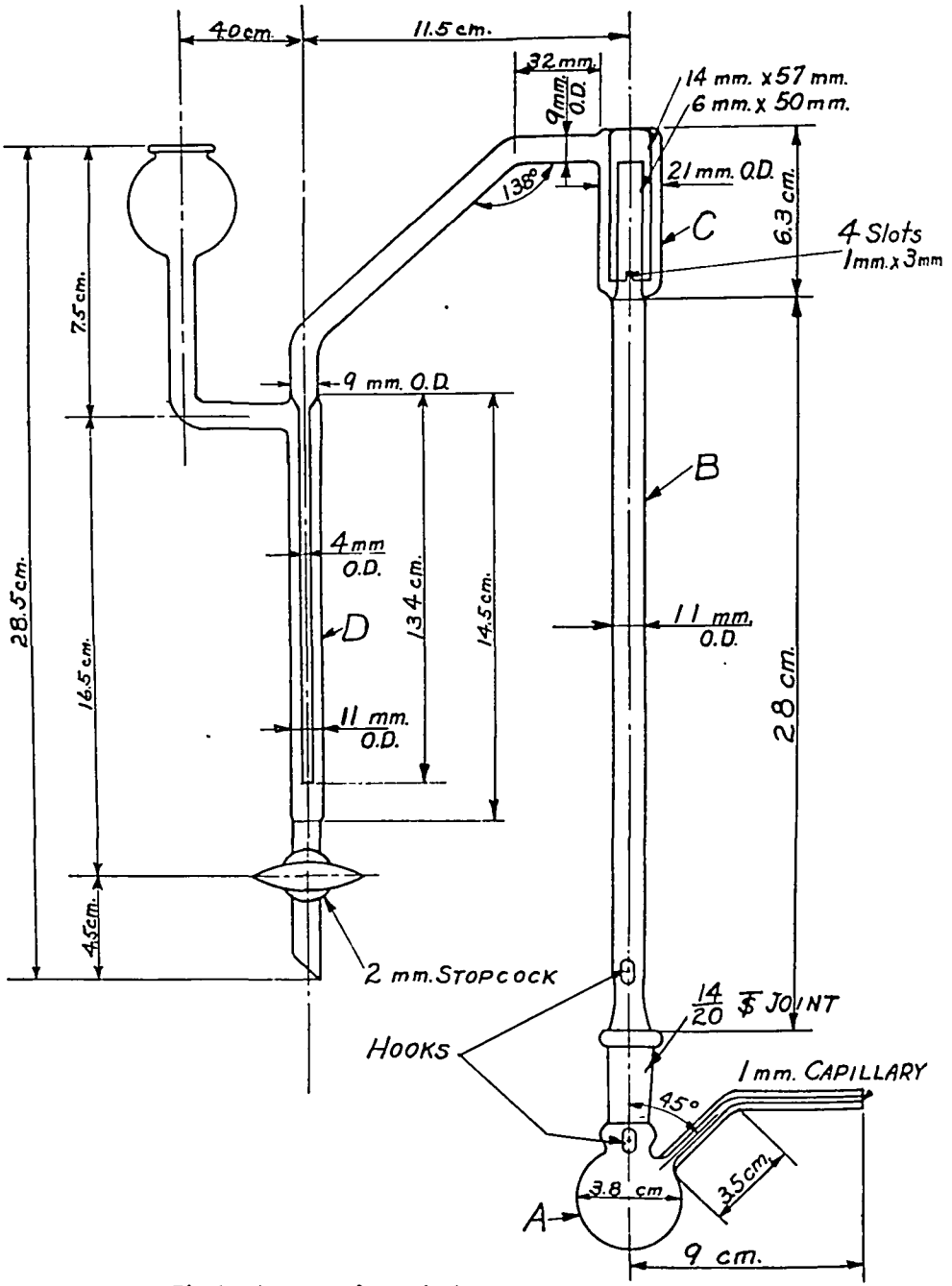

Fig. 1.—Apparatus for semi-micro determination of alkoxy groups.

add 6 to 7 cc. of the bromine-potassium acetate-acetic acid solution to the receiver. Weigh carefully a sample of the powder from the capsules equivalent to about 0.060 Gm. of acetophenetidin and contained in a gelatin capsule (size 0) and drop into the boiling flask. Add a few small glass beads and chips of porous porcelain plate and finally 6 cc. of the constant boiling hydriodic acid. Immediately attach the flask to the condenser, using a few drops of the acid to seal the joint. Bubble a slow stream of carbon dioxide through the side arm of the boiling flask (2 bubbles per second are satisfactory), place the boiling flask in an oil bath at 150°, and heat for forty minutes. Wash the contents of the receiver into a 500-cc. flask containing 10 cc. of the sodium acetate solution. Di-

lute to 125 cc. with water and add reagent grade formic acid, dropwise, with swirling, until the brown color of the bromine is discharged, then add 3 additional drops. This usually requires a total of 12 to 15 drops. After standing three minutes, add 3 Gm. of potassium iodide and 15 cc. of 10% sulfuric acid. Titrate the liberated iodine with 0.1 *N* sodium thiosulfate. Run a blank determination on a capsule (size 0) containing a proportional amount of aspirin and apply the correction for the blank. Each cc. of 0.1 *N* sodium thiosulfate is equivalent to 0.002983 Gm. of acetophenetidin.

Aspirin.—Proceed as directed under Aspirin, Method A.

Caffeine.—Filter the chloroform solution retained from the aspirin assay into a tared beaker and evaporate to dryness on the water bath. Dry at 100° and weigh the total acetophenetidin and caffeine residue. From the amount of acetophenetidin which was determined by the ethoxy assay above, calculate the per cent of the labeled amount of acetophenetidin which is present in the capsule powder. From this figure calculate the amount of acetophenetidin present in the sample taken, and consequently in the total chloroform-soluble residue. Subtract this amount of acetophenetidin from the amount of total residue, to obtain the amount of caffeine in the sample taken.

Preliminary work on this method included assaying acetophenetidin alone in the ethoxy apparatus. A recovery of 98.64% was obtained. A known mixture of the 3 ingredients was prepared and a sample assayed for ethoxy content. Blanks were run on empty size 0 capsules and on a capsule containing amounts of aspirin and caffeine proportional in the capsule powder to 60 mg. of acetophenetidin. A slightly larger correction is required for the aspirin-caffeine blank. A blank was also run on a capsule containing 15 mg. of caffeine. This figure agreed with the blank figure obtained when an empty capsule was run. Apparently from the aspirin present is formed, by reduction, some ethyl iodide which is carried over and determined.

The recovery of acetophenetidin was 99.34%, allowing for the empty blank, and 98.44%, allowing for the aspirin blank. Since this second figure agreed more closely with the results obtained in this laboratory on pure acetophenetidin, alone, the aspirin blank was used to calculate the recovery of acetophenetidin in the analysis of commercial capsules. One determination of acetophenetidin which also contained 18 mg. of starch was run. The recovery was 98.42%, which indicates that this filler in the capsules does not affect the apparent ethoxy content.

An attempt was made to get away from the necessity of a blank by weighing the chloroform-soluble residue (acetophenetidin and caffeine) and redissolving in a minimum quantity of chloroform and transferring to the flask of the ethoxy apparatus. The chloroform was then carefully distilled off and the ethoxy determination conducted as before. When this was done there was always a loss of about 2 or 3 percentage units of acetophenetidin, however. It is difficult to account for such a loss, but probably part of it is caused by solution getting into the capillary of the flask.

The major weakness of this method is that small variations in the total chloroform-soluble residue are magnified when the per cent of caffeine is determined by difference, due to the relatively small per cent of caffeine present (Table III). However, the small amount of caffeine present was also a difficulty in the other two methods.

TABLE III.—RECOVERY OF ASPIRIN, ACETOPHENETIDIN, AND CAFFEINE FROM KNOWN MIXTURES AND COMMERCIAL CAPSULES USING METHOD C

Preparation	Recovery of Aspirin, %	Recovery of Aceto., %	Recovery of Caffeine %
Known Mixture	102.7	98.44	97.16
	101.3	98.44	95.75
Capsule A	100.6	95.07	93.73
	...	...	104.70
	...	...	101.60
	...	...	92.76
Capsule B	100.3	97.18	95.53
	...	...	92.59
	...	...	86.92
	...	...	92.17

DISCUSSION

Throughout the study of methods for the separation of aspirin, acetophenetidin, and caffeine, the caffeine has been the most difficult problem, due to the fact that it is present in the smallest quantity. Grove (7) attributes the difficulty in determining caffeine in the A. O. A. C. method to several factors: "First, the caffeine is present in the smallest proportions; second, it is the last ingredient determined and thus contains any errors of manipulative technique from the separation of the other two ingredients; third, if the hydrolysis of the acetophenetidin is not complete, any unconverted acetophenetidin will be weighed with it; and finally, extracted tablet lubricants may be weighed with the caffeine."

The question of the degree of hydration of caffeine has also been a complicating factor. Garratt (4) states: "The estimation of B. P. caffeine in galenicals can only be approximate, as the commercial article effloresces and contains varying quantities of moisture." U. S. P. XII (8) defines "Caffeine" as the hydrated form, containing one mole of water, but allows anhydrous caffeine to be used, provided allowance is made for the water content. That caffeine effloresces in air is mentioned. U. S. P. XIII states: "Caffeine is anhydrous, or contains nor more than 8% of water of hydration."

Waters (9) has made a study of the extent of hydration of caffeine, finding that in most cases the hydrous caffeine contains $^5/_6$ mole of water. He found that anhydrous caffeine showed no appreciable loss on drying at 100° for several hours, which would

equivalents, 3.30 cc. When saponified as above, an additional 1.79 cc. of alkali was consumed.

Tenuigenin A diacetate: Twelve hundredths gram of tenuigenin A was warmed on the water bath for five hours with 2 cc. of acetic anhydride and a trace of pyridine. When cooled to the room temperature, the reddish solution was treated with a sufficient quantity of powdered ice under shaking. The diacetate separated out at first as an oil which solidified on standing. It was taken up with ether in which it was easily soluble. The ethereal solution was dried with anhydrous sodium sulfate and distilled and the residue taken up with little ethyl acetate. On addition of light petroleum ether, it crystallized out in colorless rhombic prisms, which, on exposure to air, became opaque and fell into a white powder, m. p. 280°. The analytical data indicated the compound to be a diacetyl derivative.

Anal.—Calcd. for $C_{27}H_{38}O_8(CH_3CO)_2$: C, 64.58; H, 7.70. Found: C, 64.30, 64.26; H, 7.68, 7.71.

When titrated with alkali, the following results were obtained: 9.210 mg. of substance dissolved in alcohol was titrated with 0.01 *N* NaOH against phenolphthalein. Calculated for 2 equivalents, 3.20 cc., found: 3.23 cc. The mixture was then refluxed for two hours after addition of 2 cc. of 0.1 *N* NaOH and titrated back. Calculated for 3 equivalents (lactone and 2 acetyl groups), 4.80 cc.; found 4.87 cc.

Tenuigenin B: The alcoholic hydrochloric acid mother liquor of tenuigenin A obtained above was diluted with water when a voluminous precipitate resulted. It was filtered, washed with water, and dried. On repeated crystallization from a mixture of acetone and ethyl acetate, tenuigenin B was obtained as colorless fine needles, m. p. 248°. It was more soluble in acetone than tenuigenin A. When analyzed, its composition agreed with the formula $C_{30}H_{46}O_8$:

Anal.—Calcd. for the formula $C_{30}H_{46}O_8$: C, 67.42; H, 8.67. Found: C, 67.82, 67.88; H, 9.07, 8.68.

On direct titration with alkali against phenolphthalein, 7.78 mg. of the substance dissolved in 2 cc. of alcohol required 2.93 cc. of 0.01 *N* NaOH; calculated for 2 equivalents, 2.91 cc. Two cubic centimeters of 0.1 *N* NaOH were added and, after boiling for two hours, the mixture was titrated back. Calculated for 1 equivalent, 1.45 cc.; found, 1.32 cc.

A second titration with 6.188 mg. of substance required 2.41 cc. of 0.01 *N* NaOH instead of 2.32 cc. theoretical. On saponification as indicated above, an additional 1.28 cc. of alkali was consumed.

Tenuigenin B diacetate was prepared in a similar way as in the case of tenuigenin A acetate. When crystallized pure from a mixture of ethyl acetate and petroleum ether, it formed shining plates, m. p. 272°. When intimately mixed with tenuigenin A acetate, it melted at 262°. Unlike tenuigenin A diacetate, its crystalline form remained unchanged on exposure to the air. Analytical results showed the substance to be a diacetate:

Anal.—Calcd. for $C_{30}H_{44}O_8(CH_3CO)_2$: C, 66.02, H, 8.15. Found: C, 65.73, 65.45; H, 8.42, 8.36

Eight and twenty-eight hundredths milligrams of the substance was titrated with 0.01 *N* NaOH against phenolphthalein. Calculated for 2 equivalents, 2.68 cc.; found: 2.72 cc. After adding 2 cc. of 0.1 *N* alkali and refluxing for two hours, the mixture was again titrated. Calculated for 3 equivalents (lactone and 2 acetyl groups): 4.02 cc.; found: 3.72 cc.

SUMMARY

An amorphous saponin has been isolated from the Chinese drug, Yüan Chih which is botanically *Polygala tenuifolia* Willd. On hydrolysis with alcoholic hydrochloric acid, this amorphous principle gives rise to two crystalline sapogenins which are named tenuigenin A and tenuigenin B. Tenuigenin A has the composition $C_{27}H_{40}O_8$, melting at 272°, and tenuigenin B has the formula $C_{30}H_{46}O_8$ and a melting point of 248°. Both genins are found to be dibasic and contain in each molecule one lactone and two OH groups. Their diacetyl derivatives have been prepared.

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Assay of Heparin. I. Method and Qualitative and Quantitative Aspects Using Beef and Sheep Plasmas*,†

By R. H. K. FOSTER‡

A method of assay for heparin based on the use of frozen beef or sheep plasma is described. The clotting curves of both plasmas are essentially the same except for high concentrations of heparin for which slight differences were noted. The visual estimation of degree of coagulation is easier with beef plasma than with sheep plasma because of the greater density of the clots of the former.

AMONG the methods proposed for assaying heparin, the two most common employ beef or sheep plasma. Beef plasma has been used by Reinert and Winterstein (1) and Foster (2) and sheep plasma by Kuizenga, Nelson, and Cartland (3) and by Klein and Hier (4). Kuizenga, *et al.*, compared the two plasmas and reported that sheep plasma gives a much sharper end point than beef plasma. Their criterion for the end point is an all-or-none response, i.e., the plasma is either clotted or fluid. They observed a narrow range of coagulation in relation to dosage for sheep plasma and a wide range for beef plasma. They stated that questionable clots were rarely encountered. However, they used dosage increments of 15 to 20 per cent and present results indicate that all gradations of clotting may fall within these dosage limits not only with sheep plasma but with beef plasma as well.

In the clotting process there is an inverse quantitative relationship between the amount of fibrin formed and the amount of inhibiting substance present. This relationship is seen only beyond a certain threshhold value and the curve representing this relationship is quite similar, except for inversion, to the usual sigmoid dose-effect curves of pharmacological phenomena. When a sample is reported as simply clotted or fluid (by tilting the tube), the dose-effect characteristic is in effect a vertical line. If the end point were an all-or-none response of this type, estimation of the exact inhibiting dose would be simplified. The *apparent* all-or-none end point may actually be almost anywhere along the lower end of the dose-effect curve since a just-fluid state of the plasma as determined by tilting will often be effected by slightly varying amounts of heparin in different tests or even more by variations in the character of the fibrin deposits. Furthermore, a slight jar may cause a "clotted" sample to flow.

The clotting curve is a function of both time and heparin concentration. In assaying heparin the time factor should be constant. Then within the correct range the amount of clot formed is a definite function of the amount of heparin.

EXPERIMENTAL

Preparation of Plasma.—Blood was collected in Pyrex bottles of 2 L. capacity from freshly slaughtered beef and sheep. Marks were placed on the bottles at 1- and 2-L. capacities. Four grams of sodium citrate in 50 cc. distilled water for every 950 cc. of blood to be collected was placed in the bottles prior to collection. On removal to the laboratory within one-half to one and one-half hours (a delay usually occurred on account of government rules on meat inspection), the blood was centrifuged thirty minutes at 2000 r. p. m. in 250-cc. bottles in a No. 2 International centrifuge. The supernatant plasma was carefully drawn off by low suction through a tube constricted to about 1–2 mm. The different portions were combined and the plasma distributed in 50- or 100-cc. bottles or 500-cc. Florence Flasks. The plasma in the flasks was quick-frozen by rotating them in a freezing bath of acetone and dry ice; that in the bottles was slow-frozen by placing them in a freezing unit.[1] The plasma was kept frozen until needed. Samples of frozen beef and sheep plasma were also received from Dr. David Klein of The Wilson Laboratories and were used in part of the work. These were slow-frozen specimens.

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† This investigation was undertaken for the Committee on Revision of the Pharmacopœia of the United States.

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[1] Insulated box containing dry ice.

Assay Procedure.—The assay method consists essentially in adding a known amount of heparin to 1 cc of the citrated plasma and followed by 0.9% saline for adjustment to constant volume and finally calcium chloride. The tubes (13 × 100 mm. Pyrex test tubes) are observed for the degree of coagulation after incubating for a definite time. This follows the procedure previously described (2) except for two important modifications. These consist in the use of twice the amount of calcium chloride formerly employed and an incubation period of one hour instead of three hours.

The order of adding reagents is: plasma + heparin + saline + calcium chloride, the total volume being 2.0 cc. Table I illustrates this and gives the volumes and concentrations employed. Kuizenga and co-workers added reagents in the order: heparin + saline + plasma + calcium chloride, the total volume being 1.4 cc. and in tests their procedure was repeated.

TABLE I.—SAMPLE PROTOCOL OF AN ASSAY

Expt. 19, Test 5. Heparin No. 645, 20γ/cc. in 0.9% saline.
Beef plasma, No. 65222 (Wilson).
Thawed 1° 25′ when used.
Start: 2:25 p. m. Observed at 3:25 p. m.
Water-bath temperature 37°.

Tube No	Beef Plasma, Cc	Heparin, 20 γ/Cc Weight, γ	Volume Delivered, Cc.	Burette Reading, Cc.	Saline, 0.9% Volume Delivered, Cc.	Burette Reading, Cc.	$CaCl_2$, 10 mg./Cc. Cc.	Degree of Clotting at 1 Hr.	All-or-None End-Point 1 Hr.
1	1.0	5.2	0.26	0.26	0.54	0.54	0.2	4+	Cl
2	1.0	5.4	0.27	0.53	0.53	1.07	0.2	4+ (−)	Cl
3	1.0	5.6	0.28	0.81	0.52	1.59	0.2	+(−)	?
4	1.0	5.8	0.29	1.10	0.51	2.10	0.2	0.	Fl
5	1.0	6.0	0.30	1.40	0.50	2.60	0.2	Tr.	Fl
6	1.0	6.2	0.31	1.71	0.49	3.09	0.2	Tr.	Fl
7	1.0	6.4	0.32	2.03	0.48	3.57	0.2	Tr.	Fl

CD_{50} = 5.51 γ, obtained by plotting.
Cl = clotted; Fl = fluid; ? = doubtful.

In each test a series of 5 or more tubes was employed to cover the desired dosage range. The median dose using beef plasma was usually about 5 γ of heparin (in Table I, the median dose is 5.5 γ) and using sheep plasma about 10 γ. Each solution was placed in the entire series of tubes of the test before adding the next solution. As soon as the calcium chloride was added the tubes were stoppered with paraffined corks, inverted 4 times in 4 directions, and incubated at 37° room temperature.

Except in preliminary tests the dosage interval of heparin was 0.2 γ for beef plasma assays and 0.4 γ for sheep plasma assays when employing the author's method but ½ γ and 1 γ, respectively, when employing Kuizenga's method.

Koch microburettes were used since greater speed and accuracy can be obtained with them. The sizes found most convenient were: 10 cc. capacity for the plasma, 5 cc. capacity for the heparin solutions and saline, and 2 cc. capacity for the calcium chloride solution. All glassware was cleaned with sulfuric acid-dichromate cleaning solution followed by thorough rinsing with tap water and redistilled water (5).

Qualitative Description of Coagulation.—Although it is impractical to describe here in detail all of the variations that have been observed among various samples, a few general observations seem warranted and worth while. Many factors alter the character of the clot, whether partial or complete, and even in duplicate samples slight differences may be noted. Using identical techniques there is often considerable variation in appearance when the plasmas are of different origins. This is particularly true of beef plasma. The homogeneity of sheep plasma clots tends to obscure differences.

With fresh unfrozen material, beef plasma produces a rather dense opaque clot which almost invariably retracts on standing. The degree of retraction may vary considerably among different plasmas. Sheep plasma produces a much paler and more translucent clot which does not retract on standing. Beef plasma is yellow-orange and the clot a grayish yellow while sheep plasma is pale straw color and the clot light gray. Both clots are lighter color than their plasmas, due mainly to their greater opacities so that the color is derived from reflected light to a greater degree.

When frozen plasma is employed certain differences from fresh plasma are noted. The clotted beef plasma now fails to retract. The clots from both plasmas are lighter in color and less dense. In particular the sheep plasma clot is more highly translucent and some specimens border on transparency.

Very often after thawing the frozen plasmas a flocculent precipitate or coagulum was noted. This occurred more often and in greater abundance in sheep plasma. The coagulum interfered with the assay and filtration through glass wool was usually found necessary. With the beef plasma, a small amount of coagulum would be dispersed and was much slower in reforming than in the sheep plasma. Filtration was not usually considered necessary. Filtration always reduced the clot densities and re-

duction in density was more obvious in the sheep plasma clots. On account of this high translucency it was often difficult to assess the degree of coagulation in the sheep plasma clots.

When sufficient heparin was present to partially inhibit coagulation the coagulum in sheep plasma was nearly always homogeneous. Only in tubes containing the slighest amount of clot was there a tendency to heterogeneity in the clot formation. This followed the time-coagulation pattern of samples containing less heparin, i.e., early in the clotting process, say after five or ten minutes, many dispersed foci of coagulation were seen but as time progressed these fused to what appeared to be a uniform gel. In beef plasma there was usually little tendency to produce a uniform gel, except in complete clots (with little or no heparin). The initial foci of coagulation were maintained and often became gradually more prominent so that the final partial clot had a flocculent appearance. In addition there was a tendency to stratification. The clot density near the surface and around the walls of one tube was almost always greater than in the center. In tubes containing successively less heparin the clot in the upper portion was deeper and denser than elsewhere, often in marked contrast. Why this should be in spite of thorough mixing is unknown but apparently air-plasma surface phenomena play a role. In occasional frozen specimens of beef plasma, the reverse was the case, i.e., the focal points of clotting seemed to settle and the final state was one with an almost clear solution near the top. This was more likely to occur in plasmas from blood which stood at room temperature several hours before centrifuging.

Quantitative Estimation of Clot.—The end point in the assay is the formation of a 50% clot; i.e., it represents the conversion of half of the fibrogen to fibrin and corresponds to the average lethal dose (LD_{50}) or the average effective dose (ED_{50}) of drugs in general. Estimation of what constitutes a 50% clot is by visual inspection, judging relative densities by comparison of tubes containing partial clots with one fully clotted. The amount of heparin which results in a 50% clot is termed the 50% clotting dose or CD_{50}.

A fully clotted sample is one which is as dense as the clot in a tube containing no heparin. Such a clot is designated 4+. Partial clots are estimated as +, 2+, and 3+, representing 25%, 50%, and 75% of complete coagulation.

The estimation of what constitutes a 2+ clot is not easy and two observers would probably not agree. The same applies to the other degrees of clotting. However, if the same observer makes all the readings simultaneously, any bias is likely to be applied to both the standard and unknown in a corresponding manner and error from this source is thus largely neutralized.

The degree of clotting cannot be compared with a permanent standard. The clotting characteristics vary among plasmas, with the age of the plasma after thawing and with slight variations in technique. In each assay one must ascertain what density constitutes a 4+ clot and evaluate the various degrees of clot formation accordingly.

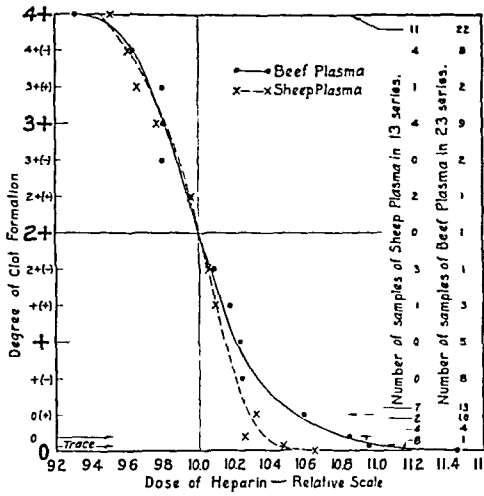

Fig. 1.—Dose-effect curves for the clotting of recalcified heparinized citrated beef and sheep plasmas The ordinate represents the degree of clot formation. The abscissa gives the *relative* dose of heparin with the CD_{50} taken as 10.0. The data employed in constructing the curves were taken only from series of tubes in which the CD_{50}'s could be estimated. There were always at least 2 points, usually above and below the 2+ coordinate, and often 3 or 4. Only the 0 or 4+ tubes nearest the curve are represented on the graph. All data were pooled on a relative dosage basis and the number of samples represented by the points on the chart are indicated in the column at the right.

The degrees of clot formation so far mentioned constitute five stages, that is, 0 or no coagulation, +, 2+, 3+, and 4+. In whole blood only major differences in the degree of clotting can be evaluated. In plasma, however, intermediate stages are recognizable. Intermediate values in the clotting scale used are represented by adding + or − signs to the above symbols. Thus, the entire clotting scale becomes 0, 0(+), +(−), +, +(+), 2+(−), 2+, 2+(+), 3+(−), 3+, 3+(+), 4+(−), and 4+. This scale is the ordinate in Fig. 1. It has been discussed in detail elsewhere (2).

Because of the greater translucency of sheep plasma clots, it is more difficult to assess the degree of clot formation than with beef plasma clots. Careful inspection is necessary by both transmitted and reflected light, against the sky or illuminated opal-glass background, and especially with side lighting against a black background.

Samples slightly coagulated are difficult to estimate and two terms have been taken to represent two degrees of slight clots, viz., "0" and "trace" The relative values of these are indicated on Fig. 1. The reason for traces of beef plasma clot appearing in several successive tubes with the higher doses of

heparin is not known. In any event the extent was found to be less than indicated by Kuizenga's data and traces appear to be unimportant as far as the assay is concerned.

It is difficult to assess clotting values slightly less than 4+. The true curves probably flatten out more before the 4+ line is reached than is shown in Fig. 1, but the slight differences in density are impossible to distinguish visually.

Evaluation of Potency.—After all tubes have been read the dose of heparin which would cause a 2+ clot (i.e., the CD_{50}) is calculated by plotting or by direct interpolation of the data.

In plotting, ordinary arithmetical coordinate paper is satisfactory. In the previous publication (2) logarithmic probability paper was employed. There was evidence that the points did not fall on an exactly straight line though the difference was usually slight. In the present method the calcium concentration (and heparin concentration as well) and incubation time are different; so a direct comparison is not valid.

By "direct interpolation" is meant the estimation of the CD_{50} directly from the data; e.g., if two doses of 4.8 γ and 5.2 γ give clotting values of 3+ and +, respectively, then the dose for the estimated 2+ clot would be 5.0 γ.

The potency of the unknown may be estimated in terms of the standard as units or per cent, i.e.:

$$\frac{CD_{50}\text{ standard}}{CD_{50}\text{ unknown}} \times \text{units/mg. standard} = \text{units/mg. unknown}$$

$$\frac{CD_{50}\text{ standard}}{CD_{50}\text{ unknown}} \times 100 = \text{per cent of standard}$$

It should be mentioned here that the end point is sharper when the larger amount of calcium is employed, i.e., the dose-effect curve is steeper. The range of dosage of heparin on the clotting characteristic is considerably greater when only 1 mg. of $CaCl_2$ is used.

RESULTS

It has not been the purpose of this paper to report in detail the data which have been obtained but only to describe the procedure and general observations. Of particular interest, however, is the summary of data showing the sharpness of the end points obtained from both beef and sheep plasma. This, already alluded to, is presented in Fig. 1.

Kuizenga, *et al.*, described a greater dosage spread in the coagulation of beef plasma than of sheep plasma. In the present experiments the extent of this dosage spread was found to be much less than they indicated. The clotting curves of Fig. 1 are almost identical except in the lower region. Here the beef plasma coagulation curve flattens out more than the curve for sheep plasma. From Kuizenga's chart the doses just giving complete inhibition and complete clotting were taken and their ratios compared with ratios of present data. The results of these calculations are shown in Table II. Good agreement is seen for the ratios with sheep plasma but not for those with beef plasma. Present results give a dosage range (in relative units) of 1:1.12 when sheep plasma is used and 1:1.23 when beef plasma is used.

TABLE II.—SHARPNESS OF END POINT

	Sheep Plasma Doses Just Giving Complete Inhibition and Complete Coagulation	Ratio	Beef Plasma Doses Just Giving Complete Inhibition and Complete Coagulation	Ratio
Kuizenga's data (3)	18 γ–16 γ	1.13	14 γ–6 γ	2.33
From Fig. 1.	10.65–9.55[a]	1.12	11.45–9.31[a]	1.23

[a] Relative doses

Calcium Concentration.—In the original method described by Reinert and Winterstein (1) the amount of calcium chloride was approximately 1 mg. per tube. The plasma was "standardized," using 2 γ of heparin (the Reinert and Winterstein anticoagulant unit) in each tube, to determine the optimum amount of $CaCl_2$ necessary to just give clotting. This was actually about half the $CaCl_2$ necessary to neutralize the sodium citrate. In using 2 mg. of $CaCl_2$ (approximately the stoichiometrical equivalent of the sodium citrate) the entire original amount of calcium of the plasma becomes available. Instead of 2 γ being the amount of heparin needed to inhibit as under the Reinert and Winterstein conditions, about 5 γ are now needed for beef plasma, and about 10 γ for sheep plasma.

As far as assays on unknowns are concerned the potency of unknowns in terms of a standard were the same when sheep plasma was employed as when beef plasma was employed. There was no apparent quantitative difference between the two plasmas in their behavior toward different samples of heparin. The data on these comparisons will be the subject of a later report.

DISCUSSION

Conditions for an assay method should be such that the greatest amount of information may be obtained from a minimum of effort, i.e., results should be accurate and easy of

duplication and the procedures as free from complex techniques as possible. In establishing an assay method the end point should be taken on the steepest part of the curve. If the clotting curve were "all-or-none," it would be vertical and the reading would be simple. However, the clotting curve is not vertical. The steepest part of the slope is approximately at the 50% point on the curve (Fig. 1). This, then, is the point that should be taken for comparison of an unknown with a standard.

Since readability of the end point is a very important aspect of the heparin assay those conditions providing this should be employed. Beef plasma gives a clot of greater density than does sheep plasma and it was found that this factor was important in being able to read clots with relative ease. Clotted fresh beef plasma is the easiest to read but the use of frozen plasma offers the advantage of being able to stock a large supply for use at any time.

Beef and sheep blood are about equally obtainable in most areas so availability is not an important factor.

It is not the purpose here to discuss other types of assay methods but a brief comment seems in order. The chemistry of heparin is not completed and it is not known just how pure commerical heparin is. A chemical method, such as the colorimetric assay using toluidine blue, would be a much more rapid procedure but like the colorimetric assay of digitalis there are objections on the grounds of the uncertain relationship between colorimetric results and anticoagulant potency. Colorimetric tests, chemical analysis. or physicochemical tests may finally prove to be adequately reliable but for the present we must depend on a bioassay.

SUMMARY

1. A procedure for assaying heparin has been described. It is based on the use of frozen beef or sheep plasma.

2. Comparisons made with sheep and beef plasma indicate the clotting curves of the two are relatively the same except for high concentrations of heparin when the curve for beef plasma flattens out slightly more than the curve for sheep plasma.

3. Comparisons of an unknown with standard are made with the dose giving a 2+ or 50 per cent clot. This is called the CD_{50}. It is estimated by plotting or interpolating the data.

4. The density of beef plasma clots is greater than those from sheep plasma and the visual estimation of the degree of coagulation is therefore easier with the former

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WHO MAKES IT?

The National Registry of Rare Chemicals, Armour Research Foundation, 33rd, Federal and Dearborn Streets, Chicago, Ill., seeks information on sources of supply for the following chemicals:

N-homoveratryl homoveratramide
Ferrous aspartate
Ferrous glutamate
Ferrous glycinate
Ferrous aminobenzoate
Benzofluorene
Benzoperylene
Benzochrysene
Dehydromucic acid
Cuskhygrine
Hygrine
2-Phosphoglyceric acid
3-Phosphoglyceric acid
Glucose-6-phosphate
Hydroxytyramine
Epinine
Citraconic acid
1-Glyceraldehyde
Creatine phosphate
d-Quercitol

Sodium Theophylline Glycinate*

By JOHN C. KRANTZ, JR., JAMES M. HOLBERT, HARRY K. IWAMOTO, and C. JELLEFF CARR

A method of preparation and the characterization of Sodium Theophylline Glycinate are described. This new combination produces a typical theophylline response in animals and it is well tolerated in large amounts in man.

IT IS WELL established that alkali salts of organic acids render theophylline water-soluble. Owing to the slightly acidic property of the principle (dissociation constant 1.62×10^{-11} at 25°) (1) it has been combined with ethylene diamine to form one of its most popular dosage forms.

Intensive therapy with the available combinations of theophylline is limited by the gastric irritation produced by the principle. Gastric hydrochloric acid readily decomposes the theophylline complexes and liberates the free amine which produces irritation to the mucosa.

It occurred to one of the authors (J. C. K.) that the use of an amino acid instead of such acids as acetic or salicylic might obviate this difficulty. Having used glycine successfully in the buffering of gastric acidity combined with aluminum hydroxide (2) we chose this amino acid.

EXPERIMENTAL

Preparation.—Sodium theophylline glycinate was prepared as follows:

One mole (198 Gm.) of theophylline U. S. P was added to 1 L. of 1 *N* NaOH (40 Gm., 1 mole). The theophylline did not completely dissolve but remained as a ropy suspension. To this mixture was added 2 moles (150 Gm.) of glycine. Solution was effected with gentle heat and then filtered to remove any suspended material. The solution was evaporated to dryness at reduced pressure and at a temperature not exceeding 70°. More drastic conditions of drying resulted in discoloration of the product. The product is a white, almost anhydrous material containing approximately 50% theophylline.

Physical Properties.—Sodium theophylline glycinate has no melting point; however, it darkens at 190° and the entire mass becomes black at 210°. It is soluble in water to the extent of approximately 18% (*w/v*). In the preparation of a saturated solution, care must be taken not to use too great an excess of the solid, for under these conditions preferential solution of sodium glycinate occurs with the resulting precipitation of theophylline. A saturated solution of the material has a *p*H of 8.7 to 9.1, a density at 20° of 1.05, and exhibits unusual stability to carbon dioxide.

Structure.—In our opinion the structure of sodium theophylline glycinate is best represented by the equilibrium:

$$C_7H_7O_2N_4Na + NH_2CH_2COOH \rightleftarrows C_7H_8O_2N_4 + NH_2CH_2COONa$$

This is concluded from the fact that solution of sodium theophylline glycinate of the same physical characteristics may be prepared from a mixture of sodium theophylline and glycine or from a mixture of theophylline and sodium glycinate. The following results illustrate this experimentally.

Four grams of theophylline were dissolved in 20 cc. of 1 *N* NaOH and diluted to 70 cc. The weights of the reactants represent equivalent amounts. The sodium theophylline formed is not completely soluble but remained as a ropy suspension. Glycine was then added in the ratio of 1/4 molar unite until the glycine-theophylline ratio was 5:1. The first addition of glycine caused the solution to clear. The *p*H of the solution was determined after each addition of glycine. The results are recorded below.

TABLE I.—THEOPHYLLINE SODIUM-GLYCINE RATIOS AND *p*H

Test No.	Molar Ratio of Glycine	*p*H
1	0	10.48
2	0.25	9.72
3	0.50	9.50
4	0.75	9.37
5	1.00	9.26
6	1.25	9.17
7	1.50	9.12
8	1.75	9.06
9	2.00	9.01
10	2.25	8.98
11	2.50	8.93
12	2.75	8.90
13	3.00	8.86
14	3.50	8.82
15	4.00	8.77
16	5.00	8.69

If the glycine concentration is plotted against *p*H, a smooth curve of diminishing *p*H results. There is no break in the curve to suggest compound or complex formation.

* Received Dec. 23, 1946, from the Dept. of Pharmacology School, University of Maryland, Baltimore, Md.

TABLE II.—LD_{50} RAT INTRAPERITONEAL INJECTION

Aminophylline				Sodium Theophylline Glycinate			
No. Rats	Mg./Kg.	Alive	Dead	No. Rats	Mg./Kg.	Alive	Dead
2	100	2	0	2	100	2	0
4	200	2	2	4	200	4	0
4	300	0	4	4	300	4	0
4	400	0	4	4	400	0	4
4	500	0	4	2	450	0	2
4	600	0	4	4	500	0	4
.	...	.	.	4	600	0	4

The solutions prepared in the foregoing studies were then compared with similar solutions prepared from theophylline and sodium glycinate. Accordingly, in an attempt to duplicate solution Test Number 5 in Table I, 2 Gm. of sodium glycinate and 4 Gm. of theophylline were dissolved in the correct volume of water. The *p*H of this solution was 9.22 as compared to 9.26 for Number 5. An additional 1.5 Gm. of glycine was added to the solution, thus making its composition similar to that of Test Number 9. The *p*H of this solution was 9.00 as compared to 9.01 for Number 9.

Thus it may be concluded from these results that the same mixture is formed with a combination of sodium theophylline and glycine as with a combination of theophylline and sodium glycinate. Since there is no evidence of compound formation, the composition is best expressed by the foregoing equilibrium equation.

Pharmacodynamic Studies

To ascertain whether or not full theophylline activity was exhibited by sodium theophylline glycinate (amino acetate) the following experiments were conducted.

LD_{50} **Rat.**—The LD_{50} of sodium theophylline glycinate was determined in the rat upon intraperitoneal injection, time period three hours, and compared with that of aminophylline. Each compound was injected in 2% aqueous solution. The results are shown in Table II.

This first approximation of the LD_{50} of the 2 compounds shows for aminophylline a value of 200 mg./Kg., and for sodium theophylline glycinate approximately 350 mg./Kg. The onset of the terminal convulsive syndrome at the same dosage levels was invariably more rapid with aminophylline than with sodium theophylline glycinate.

Blood Pressure Studies (Dog).—Dogs under ether anesthesia were injected intravenously with 2% aqueous solutions of aminophylline and sodium theophylline glycinate, respectively. Injections were made in 2 animals, alternating the 2 compounds. Aminophylline injections caused a precipitous fall in blood pressure to approximately two-thirds of the pre-injection level, with a prompt return to normal. Similar injections of sodium theophylline glycinate elicited falls in blood pressure to about 85% of the normal value. Injections of 20 to 50 mg./Kg. of ethylene diamine in aqueous solution

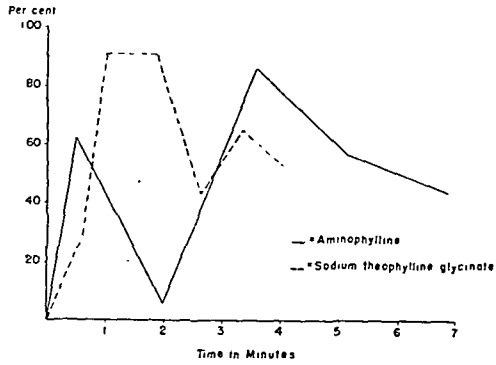

Figure 1

produced falls in blood pressure to approximately 75% of the pre-injection level. Theophylline sodium salicylate in comparable doses caused no significant change in arterial tension.

Coronary Flow Experiments (Dog).—Coronary flow experiments were carried out on the dog *in situ* by the Moravitz-Zahn technique (3). The animals were anesthetized with morphine and chlorobutanol. Heparin sodium 20 mg./Kg. was used as an anticoagulant. The right atrium was not clamped prior to the insertion of the Moravitz cannula, as rapid manipulations with sutures already in place made it possible to eliminate this feature of the experiment. The blood was collected in 5 to 20 cc. volumes and returned immediately through a Liebig condenser at 37° to the circulation through the jugular vein. The carotid blood pressure was recorded.

Aminophylline and sodium theophylline glycinate, respectively, were injected intravenously in 10 cc. volumes of 2% solution. Typical responses from the experiments on 4 dogs are shown on a percentile basis in Chart I. It is evident from the data in Chart I that each compound elicits acute coronary dilatation of approximately the same magnitude and duration.

Therapeutic Studies.—In an eighteen-month clinical trial with sodium theophylline glycinate, W. D. Paul (4) used this dosage form of theophylline in more than 300 patients. It elicited a typical theophylline therapeutic response. It was tolerated without nausea or vomiting in quantities up to 4 Gm. in twenty-four hours; i.e., 2 Gm. of theophylline. A detailed account of these studies will appear in a subsequent communication to another journal.

SUMMARY

1. A new simple theophylline combination has been prepared, namely, sodium theophylline glycinate.

2. Sodium theophylline glycinate is soluble in water, 1 part in 5 parts. Its solutions are slightly alkaline and stable in the air.

3. Sodium theophylline glycinate elicits a typical theophylline response in animals and is tolerated in unusually large amounts in man.

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Chemical Examination of *Daemia extensa* R. Br. I.*

By ASHUTOSH DUTTA and SUDHAMOY GHOSH

A chemical study of *Daemia extensa* is described. Three sterols were isolated in pure form and characterized. In addition, a glycosidic bitter principle with pituitrin-like action is described. Other minor constituents are described briefly.

DAEMIA *extensa* R. Br. (syn. *Pergularia extensa* N. E. Br.) belongs to the Natural Order Asclepiadaceae, and grows throughout the hotter parts of India, often at an altitude of 3000 feet, and in Afghanistan and Ceylon. It is a perennial twining herb with much milky juice and is foetid when bruised.

Leaves.—The leaves are thin, broadly ovate or suborbicular, acuminate, glabrous, more or less shortly pubescent above, usually velvety pubescent beneath, the margins ciliate, base deeply cordate, flowers greenish yellow or dull white, calyx pubescent divided to the base. The plant is known in Sanskrit as Chandal Dudhika or Yugmaphala, in Bengal as Chaggalbati, in Hindi as Jutak or Zitran, in Bombay as Utrani, and in Punjab as Karial.

Uses in India.—Many medicinal properties are attributed to it in the Indian indigenous systems of medicine. It is said to be pungent, cooling, anthelmintic, laxative, antiperiodic, useful in eye troubles, urinary discharges, leucoderma, uterine complaints, stranguary, inflammations, and in facilitating parturition. The juice of the leaves is used as an expectorant in the treatment of catarrhal affections. It is also given in asthma and applied to rheumatic swellings. In combination with lime or ginger it is used in amenorrhea and dysmenorrhea. All the parts of the plant are used in medicine.

Dymock (1) in his Pharmacographia Indica mentions the presence of an alkaloid which he designated as "daemine." Hartwich (2) mentions the presence of a bitter glucoside, but no details about its properties or its pharmacological action are stated in literature. As the drug finds such an important place in the Indian indigenous systems of medicine, a detailed chemical investigation was found desirable in order to find its important constituents. The material employed was the entire plant consisting of the roots, stems, leaves, and fruits. The plant was collected locally and identified by a botanist. The fresh plant when dried in the air lost about 86 per cent of its weight. The dried plant was coarsely powdered and used for all the experiments.

* Received Oct. 11, 1946, from the Department of Chemistry, School of Tropical Medicine, Calcutta, India.

EXPERIMENTAL

Test for Alkaloids.—Ten grams of the powdered drug was thoroughly extracted with boiling alcohol, the solvent removed, and the residue extracted with 1% HCl. The acid aqueous extract was filtered and the filtrate tested with alkaloidal reagents. No alkaloid, as suggested by Dymock and Hooper, could, however, be detected

Preliminary Examination.—One hundred grams of the powdered drug was extracted successively with different solvents in a Soxhlet apparatus. The extracts were freed from solvents, dried at 100°, and weighed with the following results: Petroleum ether (b. p. 40–60°) 2.82%, sulfuric ether 1.35%, chloroform 0.63%, ethyl acetate 0.67%, and alcohol 4.43%; total extract 9.88%. Each of the above extracts was tested as follows: (*a*) Petroleum ether extract: neutral in reaction and tasteless; contains fats, fatty oils, and sterols. (*b*) Sulfuric ether extract: acid to litmus; does not give any reaction with ferric chloride; bitter in taste, and gives reactions for carbohydrates. (*c*) Chloroform extract: acid to litmus, tastes bitter and contains fatty substances. (*d*) Acetic ether extract: acid to litmus, tastes extremely bitter; ferric chloride produces a dirty green coloration to its aqueous solution, and shows strong carbohydrate reaction. (*e*) Alcohol-soluble extract: acid to litmus, tastes extremely bitter; aqueous solution gives a dirty green precipitate with ferric chloride; carbohydrate reaction strongly positive and it contains inorganic salts.

Detailed Investigation.—Six and one-half kilograms of the powdered drug was extracted with several changes of boiling rectified spirit until the alcohol extracted no more of the bitter substance. Usually, 6 to 7 extractions sufficed. The combined alcoholic extracts were filtered, most of the solvent distilled off under ordinary pressure, and finally evaporated to dryness under reduced pressure. The residue which was a dark greenish semisolid mass weighed 403 Gm., equivalent to 6.20% of the crude drug. This was extracted several times with hot water and subsequently with boiling water until the extract was almost free from any bitter taste.

Examination of the Water-Insoluble Portion of the Alcoholic Extract.—The greenish black water-insoluble residue was dried and extracted repeatedly with petroleum ether in which the major portion of the residue was found to dissolve. The petroleum ether extract was filtered, the ether distilled, and a dark colored semisolid residue was obtained. Two hundred grams of this residue, equivalent to 3.5 Kg. of drug was boiled with 1 L. of 1 *N* alcoholic KOK for six hours for complete saponification. The saponified product was diluted with water, the alcohol removed by distillation, and the greenish emulsion extracted repeatedly with sulfuric ether. The ethereal extracts were washed with water, dehydrated with anhydrous sodium sulfate, and the ether distilled off. The residue which was of a bright yellow color was crystallized from absolute alcohol and the crystals weighed 63 Gm., corresponding to 1.8% of the drug. It was further purified by fractional crystallization from alcohol when the following 3 sterols were obtained in pure condition. Besides these sterols, a low-melting yellow solid mass having properties similar to sterols was obtained; attempts to purify this further were unsuccessful.

Sterol A.—Crystallized from alcohol in fine white silky needles, m. p. 172.6°; freely soluble in petroleum ether, sulfuric ether, chloroform, and benzene; moderately soluble in acetone; sparingly soluble in cold alcohol and in acetic ether, more soluble in these hot solvents; insoluble in water; specific rotation $[\alpha]_D^{26°} = +70°$ (in chloroform); gives color reactions for sterols; acetyl derivative, crystallized from ethyl acetate, melts at 203°; benzoyl derivative melts at 206°.

Sterol B.—Crystallized from alcohol in fine silky needles, m. p. 163°; freely soluble in petroleum ether, sulfuric ether, chloroform, and benzene; soluble in ethyl acetate, sparingly soluble in alcohol, more soluble in hot alcohol; insoluble in water; specific rotation $[\alpha]_D^{26°} = +60.5°$ (in chloroform); gives color reactions for sterols; acetyl derivative melts at 194.5° and benzoyl derivative melts at 217°.

Sterol C.—Crystallized from a mixture of alcohol and acetic ester in fine needles, m. p. 157°; freely soluble in petroleum ether, sulfuric ether, chloroform, and benzene; partially soluble in cold alcohol and ethyl acetate, more soluble in hot; insoluble in water; gives color reactions for sterols; acetyl derivative melts at 172° and benzoyl derivative melts at 230°.

Sterol D.—A soft yellow mass, m. p. 76–80°· soluble in sulfuric ether, petroleum ether, chloroform and benzene; partially soluble in cold alcohol and in ethyl acetate, more soluble in hot; insoluble in water; acetyl derivative melts at 211° and benzoyl derivative melts at 248–250°.

The saponifiable matter remaining in aqueous solution after extraction with sulfuric ether was precipitated by dilute sulfuric acid. The precipitate was washed, dried, and dissolved in alcohol from which only a low-melting soft crystalline substance and oily substances could be isolated.

Examination of the Resinous Matter Insoluble in Water and in Petroleum Ether.—The dark colored crude resinous substance undissolved by water and petroleum ether was found to possess a bitter taste. It was thoroughly dried and 50 Gm. of the dried material was dissolved in the minimum quantity of absolute alcohol. The solution was mixed with purified sawdust and packed in a Soxhlet apparatus. It was then extracted successively with the following organic solvents which extracted as follows: petroleum ether 0.696%, sulfuric ether 12.99%, chloroform 15.90%, ethyl acetate 10.86% and alcohol 19.09%. The chemistry and pharmacological action of these resin fractions are awaiting further study.

Examination of the Water-Soluble Fraction of the Alcoholic Extract.—The aqueous extract, after separation of the fatty and resinous matter, was concen-

trated to a small bulk under reduced pressure and allowed to crystallize. The crystals which separated out were filtered off and the mother liquor on further concentration gave another crop of crystals. These crystals weighed 156 Gm., equivalent to 2.40% of the crude drug; they were purified further by recrystallization and were found to consist of a mixture of potassium nitrate and potassium chloride, chiefly the former. The concentrated mother liquor was shaken with petroleum ether to remove fatty substances and then evaporated to dryness *in vacuo* The brown sticky residue was extracted repeatedly with hot amyl alcohol until the whole of the bitter substance passed into solution. The residue insoluble in amyl alcohol was found to contain sugars and mineral salts The amyl alcoholic extract was washed with a little water and the solvent distilled off under reduced pressure The dark brown residue was dissolved in a small quantity of water, filtered from tarry impurities, and the clear filtrate evaporated to dryness *in vacuo*. The dry extract containing the bitter principles was dissolved in absolute alcohol, in which it was completely soluble, filtered, concentrated to a small bulk and allowed to crystallize. No crystals came out even on keeping for several days and it was therefore evaporated to dryness. It weighed 21.46 Gm., corresponding to 0.33% of the crude drug.

The dry bitter substance was repeatedly extracted with sulfuric ether, the ethereal extracts washed with a little water, dried with anhydrous sodium sulfate and the solvent distilled off. The residue was dissolved in the minimum quantity of alcohol and allowed to crystallize, but as no crystals separated out, it was evaporated to dryness and finally dried in a vacuum desiccator. This residue was bitter to taste and was designated as *Bitter Principle A*.

The residue left after repeated extraction with sulfuric ether was next extracted repeatedly with chloroform in which a bitter substance came out. This fraction was designated as *Bitter Principle B*. The residue left after repeated extraction with chloroform was dissolved in absolute alcohol in which it was completely soluble. No crystals, however, separated out even on keeping it for several days. The solvent was therefore removed and the residue dried in a vacuum desiccator. This fraction is designated as *Bitter Principle C*. This was found to be the most bitter out of the 3 fractions. It was also completely soluble in water, the other two being only partially soluble.

The bitter principle C was a deep brown glassy mass and very hygroscopic in nature. When hydrolyzed with a dilute mineral acid, the hydrolyzed product strongly reduced Fehling's solution, showing its glucosidic nature. Further detailed chemical study is being pursued for a future communication.

Pharmacological Action of the Bitter Principles.—The bitter principle A was found to be practically inactive pharmacologically. The bitter principle B was found to be somewhat toxic, and the bitter principle C was found to be the most active pharmacologically. It has been recently studied in detail by Gupta and Roy (3) and a short summary of their findings is given here: The glucosidic bitter principle is toxic to white mice, frogs, cats, and guinea pigs It has a stimulant action on the involuntary muscles, plain or striated, and a pronounced effect on the circulatory system, raising the arterial blood pressure appreciably. Its action on the uterus appears to be comparable with that of pituitrin, for which it may prove to be a possible substitute. Its effects appear to be due to (*a*) the direct stimulation of the involuntary muscles, and possibly (*b*) the stimulation of the post-ganglionic cholinergic nerves in the structures concerned.

SUMMARY AND CONCLUSIONS

The plant *Daemia extensa* R. Br. was found to contain some sterols, three of which were obtained in a pure condition and the fourth in a fairly pure state. No alkaloid was found as reported by previous workers. About 2.4 per cent of inorganic salts, consisting mainly of potassium nitrate and potassium chloride, were also isolated. Besides these there was a bitter resin and three bitter principles one of which was glycosidic in nature with a strong physiological action, the use of which as a substitute for pituitrin is indicated.

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An Evaluation of Adrenochrome and Iodoadrenochrome Based on Blood Sugar Levels in Rabbits*

By FRED H. SNYDER, ERNST LEVA, and FRED W. OBERST

The evidence indicates that adrenochrome and iodoadrenochrome are ineffective in lowering the blood sugar level of normal rabbits, potentiating the effect of insulin, or neutralizing the action of epinephrine.

THERE HAS BEEN interest in the physiological importance of epinephrine oxidation products for a number of years. Green and Richter (1) obtained crystalline adrenochrome by oxidation of epinephrine with catechol oxidase and rapid evaporation of the solution. Veer (2) obtained the same product in 30–35 per cent yield, using silver oxide as the oxidizing agent; a similar method was also used by Ellis (3). Buchnea (4) recently reported preparation of stable adrenochrome by a modification of Veer's procedure. Methods for the preparation of iodoadrenochrome [Richter and Blaschko (5)] and bromoadrenochrome [Green and Richter (1)] have also been described in the literature.

The antipressor effect of adrenochrome and iodoadrenochrome in experimentally hypertensive cats has been adequately demonstrated by Oster and Sobotka (6), but the influence of these compounds on blood sugar has not been so clearly established. A report by Greiff and Stöcklein (7) indicated that adrenochrome is beneficial in the maintenance of diabetic patients. According to the interview with Buchnea (4), Marquardt has also obtained favorable clinical results in diabetes with adrenochrome; the compound apparently does not replace insulin but decreases the amount of insulin required. On the other hand, Martin (8), using a solution which probably contained adrenochrome prepared by oxidation of epinephrine with potassium permanganate, obtained slight increases in blood sugar after intravenous administration into anesthetized cats.

On the basis of these reports, it was felt that further investigation of the influence of adrenochrome on blood sugar levels was warranted. For this work we were fortunate in obtaining a small quantity of adrenochrome manufactured in Germany, presumably under the direction of Buchnea.[1] In addition, adrenochrome and iodoadrenochrome prepared in this laboratory were used in the studies.

METHODS

Preparation of Adrenochrome.—The procedure outlined by Buchnea (4) was followed as closely as possible in the preparation of adrenochrome. Because of doubtful stability of the product, the material was prepared in small quantities.

A 275-mg. sample of *l*-epinephrine was dissolved in a mixture of 7.5 cc. of absolute methanol and 0.15 cc. of glacial acetic acid. To this was added 1 Gm. of silver oxide, and the mixture was shaken for one minute. The solution, which rapidly became deep red in color, was immediately filtered by suction, and the residue was washed with 1.5 cc. of methanol. The filtrate was cooled to −10° for one hour, and the crystallized oxidation product was removed by filtration and was dried under vacuum. The yield in various runs ranged from 60 to 100 mg.

Buchnea (4) reported a melting point of 130° for his product. The material prepared in this laboratory showed signs of decomposition at about 125° but had no sharp melting point. In our hands the adrenochrome received from Germany behaved similarly.

Preparation of Iodoadrenochrome.—Iodoadrenochrome was prepared by the method of Richter and Blaschko (5). *l*-Epinephrine, in dilute acetic acid, was oxidized by potassium iodate. The dried material, obtained as crystals on cooling the reaction solution, was used without further purification.

Animal Experiments.—Adult albino rabbits, weighing about 2.5 Kg., were used in all tests; the effects of individual variation were eliminated as far as possible by rotating the animals on the various experiments with an interval of at least a week after each test.

Adrenochrome and iodoadrenochrome were administered orally, subcutaneously, or intravenously in doses ranging from 2.5 mg. to 40 mg. In one set of experiments the compounds were administered

* Received Jan. 16, 1947, from the Department of Biochemistry, Research Laboratories, The Wm S Merrell Co., Cincinnati, Ohio.

[1] This material was generously supplied by Dr. Charles E. Reed, Assistant Chief of the Chemical Unit, Technical Industrial Intelligence Branch, Department of Commerce, Washington, D C.

Biochemistry of Cancer, by JESSE P. GREENSTEIN. Academic Press, New York, 1947. viii + 389 pages. 15 x 23 cm. Price $7.80.

Anyone who has ever attempted to follow the literature of cancer research will testify to its confusing, amorphous character. Dr. Greenstein is apparently quite aware of this for in the first chapter of his book he states, "That which is fine and enduring in this literature should be separated and accorded recognition." This is precisely what the author has done and done well. He has created a bibliography which appears to give a direction to the biochemists role in the oncological sciences without adhering to a preconceived philosophy.

After an introductory chapter and a chapter on taxonomy, an extensive chapter on the extrinsic carcinogenic factors appears. Well over 100 compounds are tabulated in this chapter and 213 references are cited. The following chapter deals with the intrinsic factors such as the sex hormones responsible for tumor induction.

Attempts at control of tumor induction and of tumor growth are covered in 3 chapters dealing with nutrition, endocrinology, and chemotherapy. Included in this last chapter is a brief summary of research done with radioisotopes.

The author devotes 140 pages to the chemistry of tumors. Comparison is made between normal tissue and neoplastic tissue and much emphasis is placed on enzymology. Especially helpful in this section are the many tables. The succeeding chapter on the chemistry of the tumor-bearing host is a natural companion to the chapter on the chemistry of tumors.

The final chapter is a summary of the present status of the problem. Those who read this monograph expecting to find an answer to the cancer problem will be disappointed, for the author clearly states that the basic truths are still unknown. However, the author believes that "one of the principal hopes of the control of cancer lies fundamentally in a knowledge of the chemical nature of the malignant cell, and in successful development of chemotherapeutic approaches which must depend upon this knowledge."

After the enormous success of collaborative research culminating in the atomic bomb, many people have expressed the same hope for cancer research and the lay press has often echoed the hope that the investment of enough money is all that is needed to solve this cruel problem. Such persons do well to mark Dr. Greenstein's statement: "The control of cancer is not like the development of the atomic bomb which was an application of discoveries already made, but rather is contingent upon the discovery of biological and chemical principles inherent in living matter, and which for the most part are still unknown."

No pharmaceutical research library interested in cancer research should be without this monograph. The author has so successfully separated the "fine and enduring" that many valuable hours of library research will be saved the research worker. At the same time the author's style is so pleasing that many will enjoy reading this monograph for background.—MELVIN W. GREEN.

The Pharmaceutical Industry, by ROBERT A. HARDT. Bellman Publishing Company, Boston, 1946. 32 pages. 15 x 23 cm., paper bound. Price $1.00.

This little brochure is one of a series of 75 monographs dealing with the character of occupations and designed to be used in guidance activities. The pharmaceutical industry is sufficiently diversified that it is difficult to describe its possibilities without speaking of average conditions. Mr. Hardt has done a good job in describing the personal qualifications of the potential employee, the academic training needed, the salary scales, chances for advancement and the like. The statements are as factual as can be expected in a booklet where generalities must be used.

It is unfortunate that such professional associations as the American Chemical Society, the American College of Apothecaries, the American Institute of History of Pharmacy, AMERICAN PHARMACEUTICAL ASSOCIATION, and American Society of Hospital Pharmacists are listed as trade associations. The reviewer believes also that some of the periodicals listed do not give a very representative picture of the pharmaceutical industry.

Pharmacy Colleges will find this booklet useful in many ways.—MELVIN W. GREEN.

Scientific Edition

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Studies in Chemotherapy. IV. The Synthesis of Certain Unsymmetrical Disulfide Derivatives of the 4-Hydroxyphenyl Radical*,†

By SVERRIR MAGNUSSON‡, JOHN E. CHRISTIAN, and GLENN L. JENKINS

A method for the synthesis of several unsymmetrical diaryl disulfides having the 4-hydroxyphenylsulfenic radical or its acetyl or benzoyl derivatives as one of the components is described.

THE ACTIVITY of a few symmetrical disulfides against some kinds of microorganisms (1) is the basis for the synthesis of some unsymmetrical diaryl disulfides described in this work. The types of compounds herein described are limited to structures having the 4-hydroxyphenylsulfenic radical or its acetyl or benzoyl derivatives as one of the components. For the synthesis of starting materials the following methods were used.

A compound claimed to be bis(4-hydroxyphenyl) disulfide (2) was made by the reaction of sulfur with phenol under refluxing in a solution of sodium carbonate in glycerol. The method did not result in the formation of the claimed compound.

Diazotized aminophenol was reacted in the cold with potassium ethylxanthate to form the xanthate ester which upon heating and following hydrolysis with alkali yielded 4-hydroxythiophenol.

The same compound was obtained by forming the carbethoxy ester of sodium 4-hydroxyphenyl sulfonate, and following treatment of the dry sodium salt with phosphorus pentachloride, the obtained sulfonyl chloride was reduced with zinc and hydrochloric acid, and the resulting carbethoxy thiophenol was hydrolyzed with alkali to the 4-hydroxythiophenol.

By an analogous method 4-benzoxythiophenol was made. The sodium 4-hydroxyphenyl sulfonate was benzoylated; the dry sodium salt was treated with phosphorus pentachloride which, following reduction of the obtained sulfonyl chloride, yielded 4-benzoxythiophenol. This compound was

* For previous papers in this series see THIS JOURNAL, 35, 328–35(1946).

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‡ Eli Lilly and Company Research Fellow.

oxidized to bis(4-benzoxyphenyl) disulfide. The same compound was obtained by oxidation of 4-hydroxythiophenol with ferric chloride to bis(4-hydroxyphenyl) disulfide followed by esterification with benzoyl chloride. The bis(4-acetoxyphenyl) disulfide was obtained by acetylation of bis(4-hydroxyphenyl) disulfide with acetic anhydride.

The 4-acetoxy- and 4-benzoxyphenylsulfenyl chlorides, respectively, were prepared by the action of dry chlorine on the corresponding diesters of bis(4-hydroxyphenyl) disulfide dispersed in an anhydrous indifferent solvent.

The unsymmetrical disulfides were prepared as follows: By the reaction of 4-benzoxyphenylsulfenyl chloride with thiophenol and *o*-, *m*-, and *p*-thiocresols and 4-hydroxythiophenol, respectively, there were obtained 4-benzoxydiphenyl disulfide, 4-benzoxy-2′-methyldiphenyl disulfide, 4-benzoxy 3′-methyldiphenyl disulfide, 4-benzoxy-4′-methyldiphenyl disulfide, and 4-benzoxy-4′-hydroxydiphenyl disulfide. By the reaction of 2- and 4-nitrophenylsulfenyl chloride, and anthraquinone-2-sulfenyl chloride, respectively, there were obtained 2-nitro-4′-hydroxydiphenyl disulfide, 4-nitro-4′-hydroxydiphenyl disulfide, and 4-hydroxyphenyl-2-anthraquinone disulfide. The reactions of phenylsulfenyl chloride, 2-chlorobenzylsulfenyl chloride, 2,4-dichlorobenzylsulfenyl chloride, and 4-benzamido-3-chlorophenylsulfenyl chloride, respectively, with 4-hydroxythiophenol failed to yield the expected compounds.

The reaction of 2-chlorobenzylsulfenyl chloride and 4-benzamido-3-chlorophenylsulfenyl chloride, respectively, with 4-benzoxythiophenol yielded compounds which have not as yet been identified. The reaction between the 4-benzoxythiophenol and 2,4-dichlorobenzylsulfenyl chloride, and 2- and 4-nitrophenylsulfenyl chlorides, respectively, failed to yield the expected compounds. By the reaction of 4-acetoxyphenylsulfenyl chloride with 4-benzoxythiophenol the 4-acetoxy-4′-benzoxydiphenyl disulfide was obtained. The reaction of the same sulfenyl chloride with *p*-thiocresol did not result in the desired compound.

By benzoylation with benzoic anhydride in an anhydrous pyridine medium there were obtained 2-nitro-4′-benzoxydiphenyl disulfide and 4-nitro-4′-benzoxydiphenyl disulfide from 2-nitro- and 4-nitro-4′-hydroxydiphenyl disulfides, respectively, and 4-benzoxyphenyl-2-anthraquinone disulfide from 4-hydroxyphenyl-2-anthraquinone disulfide.

By acetylation with acetic anhydride the 2-nitro-4′-acetoxydiphenyl disulfide was obtained from 2-nitro-4′-hydroxydiphenyl disulfide.

EXPERIMENTAL

1. A compound claimed to be bis(4-hydroxyphenyl) disulfide was made according to the procedure of Lefevre and Desgrez (2). No analysis is reported by the authors but a melting point of 116° is listed. Recrystallized from alcohol the obtained compound melted at 119–120°.

Anal.—Calcd. for $C_{12}H_{10}O_2S_2$: S, 25.61%. Found: S, 29.69%.

2. **4-Hydroxythiophenol.**—This compound was made by the Leuckart reaction (3) modified by Miller and Read (4). Yield 46 Gm. (36.5%, starting with 1 mole of 4-aminophenol and 1.4 mole of potassium ethyl xanthate), collected over a distilling range of 105–110° at 1-mm. pressure.

The same compound was more conveniently obtained according to a method described by Zincke and Ebel (5) by the following steps.

3. **Sodium 4-hydroxyphenyl Sulfonate.**—Ten moles of crystalline phenol were sulfonated and converted to the sodium salt according to a procedure of Paul (6). Yield 1580 Gm. (80%).

4. **Carbethoxy Ester of Sodium 4-Phenol Sulfonate.**—Two moles of sodium 4-hydroxyphenyl sulfonate were esterified with ethylchlorocarbonate (5). Yield 208 Gm. (73%).

5. **4-Carbethoxysulfonyl Chloride.**—One mole of the carbethoxy ester of sodium 4-phenol sulfonate was converted to the sulfonyl chloride through treatment with phosphorus pentachloride (5). Yield 224 Gm. (85%); m. p. 75–76° (from glacial acetic acid).

6. **4-Carbethoxythiophenol.**—Two-tenths mole of the sulfonyl chloride was reduced with zinc and hydrochloric acid (5). Yield 22.5 Gm. (56.6%). The authors report up to 70% yield from this step.

7. **4-Hydroxythiophenol.**—Two-tenths mole of the 4-carbethoxythiophenol was hydrolyzed with 2 *N* sodium hydroxide (5). Yield 17.5 Gm. (69.4%) collected over a distilling range of 112–117° at 2-mm. pressure. The authors do not report the yield from this step.

The 4-benzoxythiophenol was made by the following reactions:

8. **Benzoyl Ester of Sodium 4-Hydroxyphenyl Sulfonate.**—One mole of sodium 4-hydroxyphenyl

sulfonate was benzoylated with benzoyl chloride (7). Yield 233 Gm. (70%).

9. **4-Benzoxyphenylsulfonyl Chloride.**—One mole of the benzoyl ester of sodium 4-hydroxyphenyl sulfonate was converted to the sulfonyl chloride through treatment with phosphorus pentachloride (8). Yield 237 Gm. (80%); m. p. 114–115° (from glacial acetic acid). Schreinemakers (8) reports a m. p. of 115–116°.

10. **4-Benzoxythiophenol.**—Two-tenths mole of the sulfonyl chloride was added in 5-Gm. portions to a mixture of 30 Gm. of zinc dust, 150 cc. of glacial acetic acid, and 10 cc. of concentrated hydrochloric acid, the mixture being kept below 60° during the addition. An additional 90 cc. of concentrated hydrochloric acid was added and the mixture was refluxed until a sample precipitated with water formed a clear solution with alkali. After filtering the solution was diluted with water and a white product was collected and dried. Yield 17.5 Gm. (74%); m. p. 74–75° (from ligroin).

Anal.—Calcd. for $C_{10}H_{10}O_2S$: S, 13.92%. Found: S, 13.65%.

11. **4-Benzoxyphenyl Thiobenzoate.**—Made by benzoylation of 4-benzoxythiophenol with benzoyl chloride. M. p. 160–161° (from benzene).

Anal.—Calcd. for $C_{20}H_{14}O_3S$: S, 9.55%. Found: S, 9.50%.

12. **Bis(4-hydroxyphenyl) Disulfide.**—Two-tenths mole of 4-hydroxythiophenol was oxidized with a dilute solution of ferric chloride (3). Yield 20 Gm. (80%). Colorless needles, melting at 151–152° (from water).

13. **Bis(4-benzoxyphenyl) Disulfide.**—Two-tenths mole of bis(4-hydroxyphenyl) disulfide was benzoylated with benzoyl chloride. Yield 3.2 Gm. (70%).

The same compound was prepared by the oxidation of 0.1 mole of 4-benzoxythiophenol at room temperature in 50% acetic acid with 30% hydrogen peroxide. Yield 19.5 Gm. (89%). White needles from benzene; m. p. 165–166°.

Anal.—Calcd. for $C_{26}H_{18}O_4S_2$: S, 13.98%. Found: S, 14.10%.

14. **Bis(4-acetoxyphenyl) Disulfide.**—Two-tenths mole of bis(4-hydroxyphenyl) disulfide was esterified with acetic anhydride (9). M. p. 90–91° (from alcohol). Leuckart (3) reports m. p. 88–89°.

15. **4-Benzoxyphenylsulfenyl Chloride.**—The procedure used was that described by Hubacher (9). On completion of the chlorination reaction excess chlorine was expelled from the sulfenyl chloride solution by bubbling dry nitrogen through the system for one-half hour. The sulfenyl chloride was not isolated due to rapid decomposition of the crystalline compound, but was used directly in solution for the reaction with different thiols.

All the other sulfenyl chlorides used in the following reactions were prepared in the same manner, starting with the corresponding symmetrical disulfides.

16. **4-Benzoxydiphenyl Disulfide.**—One-hundreth mole of 4-benzoxyphenylsulfenyl chloride dissolved in 25 cc. of dry carbon tetrachloride was added slowly at room temperature to a solution of 0.01 mole of thiophenol in 25 cc. of dry carbon tetrachloride, followed by refluxing the mixture for one-half hour. A trace of copper bronze was used as a catalyst. Spontaneous evaporation of the solvent left a compound which, recrystallized from ligroin, melted at 103.5–104.5°. Yield 2.8 Gm. (82.8%).

The same procedure was used for the other coupling reactions.

The 4-benzoxydiphenyl disulfide was also prepared by the reaction of the sulfenyl chloride (0.01 mole) in dry benzene solution with the sodium salt of thiophenol, obtained by the reaction of metallic sodium with thiophenol in dry benzene and nitrogen atmosphere. Yield of 4-benzoxydiphenyl disulfide was 2.57 Gm. (76%).

Anal.—Calcd. for $C_{19}H_{14}O_2S_2$: C, 67.71%; H, 4.14%; S, 18.93%; Found: C, 67.52%; H, 4.17%; S, 18.94%.

17. **4-Benzoxy-2′-methyldiphenyl Disulfide.**—One-hundredth mole of 4-benzoxyphenylsulfenyl chloride was reacted with 0.01 mole of *o*-thiocresol in dry chloroform. Tiny snow-white needles, m. p. 68.5–69.5° (from methanol) Yield 2.53 Gm. (70.9%).

Anal.—Calcd. for $C_{20}H_{16}O_2S_2$: C, 68.15%; H, 4.57%; S, 18.19%. Found: C, 68.10%; H, 4.85%; S, 17.97%.

18. **4-Benzoxy-3′-methyldiphenyl Disulfide.**—One-hundredth mole of 4-benzoxyphenylsulfenyl chloride was reacted with an equimolar amount of *m*-thiocresol in dry chloroform. Fine snow-white needles, m. p. 75–76° (from methanol). Yield 2.35 Gm. (66.7%).

Anal.—Calcd. for $C_{20}H_{16}O_2S_2$: S, 18.19%. Found: S, 17.97%.

19. **4-Benzoxy-4′-methyldiphenyl Disulfide.**—One-hundredth mole of 4-benzoxyphenylsulfenyl chloride was reacted with 0.01 mole of *p*-thiocresol in dry carbon tetrachloride. Fine snow-white needles, m. p. 115.5–116.5° (from alcohol or ligroin). Yield 2.73 Gm. (80%).

Anal.—Calcd. for $C_{20}H_{16}O_2S_2$: S, 18.19%. Found: S, 17.97%.

20. **4-Benzoxy-4′-hydroxydiphenyl Disulfide.**—One-hundredth mole of 4-benzoxyphenylsulfenyl chloride was reacted with 0.01 mole of 4-hydroxythiophenol dissolved in 50 cc. of anhydrous ether. A white microcrystalline compound, m. p. 129.5–130° (from methanol). Yield 2.52 Gm. (71.2%).

Anal.—Calcd. for $C_{19}H_{14}O_2S_2$: S, 18.09%. Found: S, 17.42%.

21. **4-Hydroxyphenyl-2-anthraquinone Disulfide.**—One-hundreth mole of anthraquinone-2-sulfenyl chloride was reacted with 0.01 mole of 4-hydroxythiophenol dissolved in 50 cc. of anhydrous ether. Small golden-yellow crystals, m. p. 192–193° (from glacial acetic acid). Yield 2.9 Gm. (79.6%).

Anal.—Calcd. for $C_{20}H_{12}O_3S_2$: S, 17.05%. Found: S, 17.07%.

EXPERIMENTAL

1. **Bis(4-Nitrophenyl) Disulfide.**—The procedures used were those of Wohlfahrt (5) and Bogert and Stull (6). The compound was purified according to the method described by Zincke and Lenhardt (7); m. p. 181°.

2. **4-Nitrophenylsulfenyl Chloride.**—The procedure used was a modification of the one described by Hubacher (8). A current of chlorine was bubbled through 1.54 Gm. of bis(4-nitrophenyl) disulfide suspended in 20 cc. of chloroform. In this experiment as well as in all others where arylsulfenyl chlorides are either being prepared or used in reactions, conditions must be anhydrous in order to avoid undesired by-products. The reaction was completed in twenty minutes resulting in a clear solution of 4-nitrophenylsulfenyl chloride (0.01 mole) Excess chlorine contained in the system was expelled by passing a current of dry nitrogen through the reaction mixture. The solution was used without isolating the compound because of the extreme instability of the latter.

3. **4-Nitrodiphenyl Disulfide.**—To a chloroform solution containing 1.9 Gm. (0.01 mole) of 4-nitrophenylsulfenyl chloride was added slowly a solution of 1.10 Gm. (0 01 mole) of benzenethiol in dry chloroform and the reaction mixture was refluxed for two hours, copper-bronze being used as a catalyst. Then the chloroform was evaporated off and the residue was recrystallized from hexane; yellow needles, m. p. 58–58.5°; yield 1.80 Gm. (68.4%).

Anal.—Calcd. for $C_{12}H_9O_2NS_2$: S, 24.35%; N, 5.32%. Found: S, 24.51%; N, 5.27%.

4. **4-Methyl-4′-nitrodiphenyl Disulfide.**—The procedure used was the same as that for 4-nitrodiphenyl disulfide. Starting materials were 1.9 Gm. (0.01 mole) of 4-nitrophenylsulfenyl chloride and 1.24 Gm. (0.01 mole) of 4-methylbenzenethiol. Yellow needles, m. p. 62–62.5°; yield 2.65 Gm. (95.5%).

Anal.—Calcd. for $C_{13}H_{11}O_2NS_2$: S, 23.12%; N, 5.05%. Found: S, 23.09%; N, 5.13%.

5. **2-Methyl-4′-nitrodiphenyl Disulfide.**—The procedure used was the same as that for 4-nitrodiphenyl disulfide. Starting materials were 1.9 Gm. (0.01 mole) of 4-nitrophenylsulfenyl chloride and 1.24 Gm. (0.01 mole) of 2-methylbenzenethiol. Yellow needles, m. p. 84–84.5°; yield 2.30 Gm. (82.9%).

Anal.—Calcd. for $C_{13}H_{11}O_2NS_2$: S, 23.12%. Found: S, 22.81%.

6. ***Attempted Synthesis of bis(4-Aminophenyl) Disulfide from Aniline, Lead Carbonate, and Sulfur.***—The procedure described by Lefevre and Desgres (9) was followed. Their results could not be reproduced, a gelatinous, uncrystallizable compound being obtained instead of the desired product. Lefevre and Desgres reported the compound to melt at 106°.

7. **Bis(4-Aminophenyl) Disulfide Obtained by Reduction of bis(4-Nitrophenyl) Disulfide.**—The method used was a modification of the one described by Shukla (10). To a refluxing suspension of 15.4 Gm. (0.2 mole) of bis(4-nitrophenyl) disulfide in 30 cc. of glacial acetic acid was slowly added a solution of 101.5 Gm. of stannous chloride, $SnCl_2 \cdot 2H_2O$, in 120 cc. of concentrated hydrochloric acid and the reaction mixture was refluxed for one hour. On cooling a crystalline material precipitated which was separated and dissolved in water and sufficient 20% sodium hydroxide solution added to make the solution slightly alkaline. Hydrogen peroxide (30%) was then added, dropwise, to the alkaline solution whereby the desired product separated. Recrystallized from dilute alcohol it melted at 76–77°; yield 10.9 Gm. (87.7%). Shukla reports a m. p. of 85°; other melting points have also been reported (11, 12).

8. **Bis(4-acetamidophenyl) Disulfide.**—The procedure used was that of Khmel'Nitzkaya and Mikel's (11). Starting from 24.8 Gm. (0.1 mole) of bis(4-aminophenyl) disulfide the yield was 34 Gm. (97.7%); m. p. 215–217°; other melting points have also been reported (11, 13).

9. **4-Acetamido-3-chlorophenylsulfenyl Chloride.**—A current of chlorine was passed through a refluxing suspension of 1.66 Gm. (0.02 mole) of bis(4-acetamidophenyl) disulfide in 25 cc. of chloroform. The reaction was completed in fifteen to twenty minutes, resulting in a clear solution of the desired compound (0.01 mole). Excess chlorine was expelled by passing a current of nitrogen through the solution. The solution was used as such without isolating the compound.

10. **4-Acetamido-3-chloro-4′-methyldiphenyl Disulfide.**—A chloroform solution of 1.24 Gm. (0.01 mole) of 4-methylbenzenethiol was slowly added to 2.36 Gm. (0.01 mole) of 4-acetamido-3-chlorophenylsulfenyl chloride dissolved in 25 cc. of chloroform. Copper-bronze was used as a catalyst. After one hour refluxing the chloroform was evaporated and the residue extracted several times with boiling heptane. The extracts were combined and chilled; the crystals formed were recrystallized from heptane. A white microcrystalline substance resulted, m. p. 113–114°; yield 1.6 Gm. (49.4%).

Anal.—Calcd. for $C_{15}H_{14}OClNS_2$: S, 19.70%. Found: S, 19.14%.

11. **4-Acetamido-3-chloro-3′-methyldiphenyl Disulfide.**—The procedure used was that described in Experiment 10, the reactants being 0.01 mole each of 3-methylbenzenethiol and 4-acetamido-3-chlorophenylsulfenyl chloride. A microcrystalline, white compound resulted, m. p. 93.5–94.5°; yield 0.50 Gm. (15.4%).

Anal.—Calcd. for $C_{15}H_{14}OClNS_2$: S, 19.70%. Found: S, 19.22%.

12. **4-Acetamido-3-chloro-2′-methyldiphenyl Disulfide.**—The procedure used was that described in Experiment 10, 2-methylbenzenethiol being used instead of 4-methylbenzenethiol. A white, microcrystalline compound resulted, m. p. 99–100°; yield 0.50 Gm. (15.4%).

Anal.—Calcd. for $C_{15}H_{14}OClNS_2$: S, 19.70%. Found: S, 19.02%.

13. Bis(2,4-Dinitrophenyl) Disulfide.—The procedure used was that of Fromm, *et al.* (14). Starting from 0.1 mole of 2,4-dinitrochlorobenzene a yield of 14 Gm. (71.4%) was obtained. Yellow needles from nitrobenzene decomposed without melting around 280°. Fromm reported the compound to explode at 280°.

14. 2,4-Dinitrophenylsulfenyl Chloride.—The procedure used was a modification of that described by Hubacher (8). A current of chlorine was bubbled through a hot (120–130°) suspension of 1.99 Gm. (0.02 mole) of bis(2,4-dinitrophenyl) disulfide in 25 cc. of nitrobenzene. The reaction was completed in one and one-half hours, resulting in a clear solution of the desired product (0.01 mole). Excess of chlorine was expelled by passing a current of nitrogen through the reaction mixture. This solution was used for the 4 following experiments. The compound may be isolated by evaporation of the solvent *in vacuo* and recrystallization of the residue from carbon tetrachloride; m. p. 94–95°.

15. 2,4-Dinitro-4′-methyldiphenyl Disulfide.—A solution of 1.24 Gm. (0.01 mole) of 4-methylbenzenethiol in 15 cc. of nitrobenzene was added slowly to a solution of 2.35 Gm. (0.01 mole) of 2,4-dinitrophenylsulfenyl chloride in 25 cc. of nitrobenzene, and the reaction mixture was heated on a steam bath for one hour. Both solutions were heated to 100° before they were mixed together. Then the solution was transferred to an evaporating dish and the nitrobenzene was evaporated. The residue was recrystallized from alcohol. Yellow needles, m. p. 114–115°; yield 2.5 Gm. (77.5%).

Anal.—Calcd. for $C_{13}H_{10}O_4N_2S_2$: S, 18.89% Found: S, 19.27%.

16. 2,4-Dinitro-3′-methyldiphenyl Disulfide.—The procedure used was the same as that for 2,4-dinitro-4′-methyldiphenyl disulfide except that 3-methylbenzenethiol was used instead of 4-methylbenzenethiol. Yellow needles, m. p. 74.5–75.5°; yield 1.9 Gm. (58.9%).

Anal.—Calcd. for $C_{13}H_{10}O_4N_2S_2$: S, 18.89%. Found: S, 18.72%.

17. 2,4-Dinitro-2 -methyldiphenyl Disulfide.—The procedure used was that described in Experiment 15 using 2-methylbenzenethiol instead of 4-methylbenzenethiol. Yellow needles, m. p. 100–100.5°; yield 2.0 Gm. (62.4%).

Anal.—Calcd. for $C_{13}H_{10}O_4N_2S_2$: S, 18.89%. Found: S, 18.53%.

18. 2,4-Dinitrodiphenyl Disulfide.—The procedure used was that described in Experiment 15, benzenethiol being used instead of 4-methylbenzenethiol. Yellow needles, m. p. 86–87°; yield 2.5 Gm. (81.8%).

Anal.—Calcd. for $C_{12}H_8O_4N_2S_2$: S, 20.80%. Found: S, 20.72%.

19. Bis(2-nitrophenyl) Disulfide.—The procedure used was that described by Bogert and Stull (6). Yellow needles from glacial acetic acid, m. p. 197–198°. Bogert and Stull reported 192–195°; other melting points have also been reported.

20. 2-Nitrophenylsulfenyl Chloride.—A modification of the procedure described by Hubacher (8), and the same one as that for 4-nitrophenylsulfenyl chloride, was used except that during the chlorination the reaction mixture was refluxed instead of applying no heat. This solution was used without isolating the compound for the following experiments.

21. 4-Methyl-2′-nitrodiphenyl Disulfide.—A solution of 1.24 Gm. (0.01 mole) of 4-methylbenzenethiol in 20 cc. of chloroform was slowly added to a chloroform solution of 1.9 Gm. (0.01 mole) of 2-nitrophenylsulfenyl chloride, and the reaction mixture was refluxed for one hour. The solution was then transferred to a beaker and the chloroform was evaporated. The residue was recrystallized from methyl alcohol. Yellow needles, m. p. 73.5–74°; yield 2.13 Gm. (76.3%).

Anal.—Calcd. for $C_{13}H_{11}O_2NS_2$: S, 23.12%. Found: S, 22.55%.

22. 3-Methyl-2′-nitrodiphenyl Disulfide.—The procedure used was that described in Experiment 21, 3-methylbenzenethiol being used intead of 4-methylbenzenethiol. Yellow needles, m. p. 74.5–75.5°; yield 2.32 Gm. (83.7%).

Anal.—Calcd. for $C_{13}H_{11}O_2NS_2$: S, 23.12%. Found: 22.60%.

23. 2-Methyl-2′-nitrodiphenyl Disulfide.—The procedure described in Experiment 21 was followed except that 2-methylbenzenethiol was used instead of 4-methylbenzenethiol. Yellow needles, m. p 103–103.5°; yield 2.32 Gm. (83.7%).

Anal.—Calcd. for $C_{13}H_{11}O_2NS_2$: S, 23.12% Found: S, 22.88%.

24. 2-Nitrodiphenyl Disulfide.—The procedure used was that described in Experiment 21 except that benzenethiol was used instead of 4-methylbenzenethiol. Yellow needles, m. p. 49–50°; yield 2.07 Gm. (78.6%).

Anal.—Calcd. for $C_{12}H_9O_2NS_2$: S, 24.35%. Found: S, 23.82%.

SUMMARY

1. A method for synthesizing certain unsymmetrical diaryl disulfides has been described.

2. The following unsymmetrical diaryl disulfides have been prepared: 4-Nitrodiphenyl disulfide; 4-Methyl-4′-nitrodiphenyl disulfide; 2-Methyl-4′-nitrodiphenyl disulfide; 4-Acetamido-3-chloro-4′-methyldiphenyl disulfide; 4-Acetamido-3-chloro-3′-methyldiphenyl disulfide; 4-Acetamido-3-chloro-2′-methyldiphenyl disulfide; 2,4-Dinitro-4′-methyldiphenyl disulfide; 2,4-Dinitro-3′-methyldiphenyl disulfide; 2,4-Dinitro-2′-methyldiphenyl disulfide; 2,4-Dinitrodiphenyl disulfide; 4-Methyl-2′-nitro-

diphenyl disulfide; 3-Methyl-2′-nitrodiphenyl disulfide; 2-Methyl-2′-nitrodiphenyl disulfide; and 2-Nitrodiphenyl disulfide.

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The Determination of Camphor in Solution with Phenol in a High Percentage of Light Mineral Oil*

By ERIC D. ROBINSON and S. C. WERCH

The standard hydrazone method for the determination of camphor cannot be employed directly on a solution of camphor with phenol in a high percentage of mineral oil. When a large percentage of mineral oil is present, it adheres to the hydrazone crystals and interferes with the gravimetric analysis. A method is here presented which eliminates this difficulty. This method is based on the extraction of camphor with alcohol. A weighed sample is centrifuged after first being emulsified by the addition of 95% ethyl alcohol. This procedure gives recoveries averaging 99.6%.

WHEN CAMPHOR is in an alcoholic solution such as Spirits of Camphor or in a small amount of mineral oil as in the National Formulary preparation of Camphorated Phenol (1), the standard hydrazone method for the determination of camphor can be employed directly. Direct application of this method, however, does not give accurate results for camphor when this stearoptene is associated with phenol in a large quantity of mineral oil. This is the case in many of the "Antiseptic Oils" that are available on the market. When a large quantity of mineral oil is present, it adheres to the hydrazone crystals and interferes with the gravimetric analysis.

Because our laboratory has had the task of determining camphor in a number of preparations containing camphor and phenol in a high percentage of mineral oil, it has been found necessary to devise a method for the separation of camphor from the oily base. This has been accomplished by extracting the camphor with alcohol. A weighed sample is centrifuged after first being emulsified by the addition of 95% ethyl alcohol. This procedure gives excellent recoveries.

METHOD

If a large percentage of mineral oil is present, proceed as follows: A 0.3- to 1.5-Gm. sample containing camphor, phenol, and mineral oil is weighed into a 15-ml. centrifuge tube. To this solution 1–3 ml. of 95% ethyl alcohol is added and the mixture shaken vigorously until an opaque emulsion is formed. The alcohol-camphor layer which forms is then separated from the mineral oil by centrifuging at about 3500 r. p. m. for at least three minutes. This separation procedure is best repeated about four times. The alcohol-camphor layer, after each extraction, is carefully poured into a 250-ml. volumetric flask. If a tiny drop of oil when warmed fails to give an odor of camphor, one can be reasonably

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certain that all the camphor has been extracted along with the phenol. The extract is now made to volume and gently mixed. Any oil-droplets that may appear at this time will settle to the bottom of the flask.

From the center of the flask draw four 25-ml. aliquots of the alcohol-camphor solution by means of a pipette. Empty each aliquot into a 500-ml. Erlenmeyer flask, then proceed with the assay for camphor as in N. F. VIII for Camphor Spirit. The amount of camphor is calculated from the weight of camphor 2,4-dinitrophenylhydrazone.

If "gunk" should appear floating about the flask prior to filtering the hydrazone after it is formed, it indicates that too much alcohol had been employed in the above extraction and that some of the mineral oil got into solution. This mineral oil also must be removed. This is done by decanting off the floating material and dissolving in boiling alcohol. The globules of mineral oil will settle to the bottom. Next pour off the supernatant liquid and cool in an ice bath. Then wash what hydrazine crystals might have formed into the crucible and proceed as before to wash with water and to dry to constant weight.

When only a small percentage of mineral oil is present, no emulsion will result from the addition of alcohol and separation by centrifuging will not be necessary.

TABLE I.—RECOVERY OF CAMPHOR FROM STOCK SOLUTION NO. 1

Sample	Sample,[a] Gm.	Camphor Added, Gm.	Camphor Recovered, Gm.	Recovery, %
1	0.1883	0.0282	0.0283	100.4
2	0.0385	0.0058	0.0057	98.3
3	0.0385	0.0058	0.0058	100.0
4	0.3746	0.0562	0.0559	99.5
5	1.2060	0.1809	0.1805	99.8
6	0.9910	0.1487	0.1484	99.8
			Av.	99.6

[a] Samples 1–6 are amounts of 6 different weighed samples.

EXPERIMENTAL

Recovery experiments were carried out on two stock solutions. One (No. 1), containing a large percentage of mineral oil and duplicating the general formula of many of the "Antiseptic Oils" on the market, was made up with 5% phenol, 15% camphor, and 80% light mineral oil. The other (No. 2) included a small percentage of light mineral oil and duplicated the National Formulary preparation of Camphorated Phenol (30% phenol, 60% camphor, and 10% light mineral oil). Extraction of the camphor was carried out on Stock Solution No. 1, while direct application of the hydrazone method was applied to Stock Solution No. 2.

The results of the recovery tests are shown in Tables I and II. Recoveries from Stock Solution No. 1 averaged 99.6% and were included in Table I. Table II presents the findings obtained from Stock Solution No. 2. Here the recoveries averaged 98.9%.

TABLE II.—RECOVERY OF CAMPHOR FROM STOCK SOLUTION NO. 2

Sample	Sample,[a] Gm.	Camphor Added, Gm.	Camphor Recovered, Gm.	Recovery, %
1	0.1543	0.0926	0.0913	98.6
2	0.1543	0.0926	0.0914	98.7
3	0.1543	0.0926	0.0911	98.4
4	0.1543	0.0926	0.0916	98.9
5	0.1543	0.0926	0.0919	99.3
6	0.1543	0.0926	0.0920	99.4
				Av. 98.9

[a] Samples 1–6 are amounts in 25-ml. aliquots from 1.5430 Gm. in 250 ml. of alcoholic solution at 20°.

DISCUSSION

The addition of alcohol to Stock Solution No. 2, which duplicates the National Formulary preparation of Camphorated Phenol, does not produce an emulsion, thus permitting the direct application of the hydrazone method. The mineral oil present in the sample goes readily into alcoholic solution with the phenol and camphor. This is due to the presence of only a small amount of mineral oil. In any case, no significant amount of mineral oil contaminates the hydrazone crystals.

When the hydrazone method for the determination of camphor was applied directly to solutions of camphor and phenol in a high percentage of light mineral oil, as in Stock Solution No. 1, results were about five times greater than the actual amount of camphor present. In these cases, mineral oil to a considerable degree is adhered to the hydrazone crystals, thus interfering with the gravimetric evaluation. Alcoholic emulsification and subsequent separation of the camphor and alcohol by centrifuging eliminated this interference.

CONCLUSION

A method is here presented which gives excellent recoveries of camphor when it is in solution with phenol in a high percentage of mineral oil.

Under such circumstances it is necessary to extract the camphor with alcohol before it is possible to apply the hydrazone reaction.

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Influence of Bile Salts on the Absorption of Quinine*

By U. P. BASU, S. MUKHERJEE, and R. P. BANERJEE

An explanation is offered of the mechanism of absorption of quinine from the intestine on the basis of its interaction with bile salts. Quinine reacts with an excess of sodium dehydrocholate or tauroglycocholate in alkaline medium to form a colloidal solution. When the latter is subjected to dialysis through cellophane, previously swollen with zinc chloride, both quinine and the bile acids are found to diffuse out through the membrane. Quinine dehydrocholate is only partially peptized by an excess of bile salt solution, and the resulting colloid, on dialysis, behaves similarly.

It is known that in order to maintain an adequate concentration of quinine in the blood for a sufficient length of time, oral administration of the drug is preferred to parenteral, and a daily dose of 2 Gm. (30 gr.) is usually required. But, on the average, about one-third of this quantity passes through the circulatory system and is subsequently excreted almost entirely through the kidneys. No difference is noticed in the quantity of quinine excreted, whether the alkaloid is administered in the form of a highly soluble salt (e.g., the dihydrochloride) or as a sparingly soluble salt (e.g., the sulfate) (1). The major portion of the quinine ingested is considered by some to be removed by the reticulo-endothelial cells (2) to the liver and destroyed during the metabolic processes (3, 4). The possibility that a part of the ingested quinine is lost through nonabsorption in the alimentary tract also cannot be excluded. It would be of interest to undertake an investigation to find, if possible, means by which the above fate of quinine in the system may be altered.

Kelsey and Oldham have shown (5) that destruction of quinine is catalyzed by an oxidase present in the system. The observations of Anderson, Cornatzer, and Andrews (3) that removal of the liver helps in the urinary excretion of quinine tends to indicate that the liver is the main seat of the above oxidase, and of the consequent destruction of quinine. It may be noted that, in the distribution of quinine in the different organs of the body, the highest concentration is found in the liver, as determined by Ramsden, Lipkin, and Whitley (6). The question suggests itself whether, by increasing the flow of blood in the hepatic arteries, the concentration and/or retention of quinine in the liver and, therefore, its fate in the system might be altered. It is known that bile salts stimulate the hepatic flow (7) and also have the property of keeping in solution several types of water-insoluble substances. If they exert a similar action on quinine, the ingestion of one or other bile salt along with quinine might be of considerable significance.

The administration of quinine and other cinchona alkaloids in combination with bile acids has previously been claimed in the patent literature to enhance the parasiticidal action of the drugs (8–12), but the manner in which this action is exerted by the bile acids has not been explained. Such work on the influence of a hydrochloretic on the metabolism of quinine was considered to be of importance.

In the present study, the interaction of quinine with bile acids or salts has been investigated under various conditions. Salts of tauroglycocholic acid and dehydrocholic acid have been used for these investigations, since the former is a common constituent of bile, and the latter, an oxidation product of cholic acid, is well known for its marked effect on bile secretion (13) and low toxicity (14); [*cf.* also Gilbert (15)].

EXPERIMENTAL

Preparation of Quinine Dehydrocholate.—Quinine (16.4 Gm.) and pure dehydrocholic acid (20.1 Gm.) were dissolved separately in chloroform by warming. The respective solutions were filtered and mixed with stirring. The chloroform was then evaporated on a steam bath and the residual mass triturated with absolute alcohol and finally mixed with ether. The ether was removed and the resulting powder was then refluxed with dilute alcohol

* Received Jan. 20, 1947, from the Bengal Immunity Research Laboratory, Calcutta, India.

(1:1) for about one hour (with the addition of charcoal). The suspension was then filtered and the filtrate cooled. Quinine dehydrocholate separated out and was collected. It melted at 148–150° with decomposition. The melting point was not depressed when the salt was melted in a sealed tube.

Anal.—Calcd. for quinine, 44.6%; found: 43.9%.

Solubility of Quinine Dehydrocholate.—For the determination of solubility, 100 ml. of water was saturated at 28° with the salt, the filtered solution was evaporated and the residue dried at 100° to constant weight. The solubility of quinine dehydrocholate in water at 28° was found to be 0.0964 Gm. per 100 ml. of water.

Interactions of Quinine Salts with Sodium Dehydrocholate and Tauroglycocholate.—The following experiments were performed in order to study the reaction, and the nature of the products formed:

(A) To 7 ml. of a 0.0568 *M* quinine dihydrochloride solution (*p*H = 3.14) was gradually added a 0.025 *M* solution of sodium dehydrocholate (*p*H = 9.06) until the *p*H of the resulting mixture was 8.2. The volume of sodium dehydrocholate required was 11.75 ml. A coarse precipitate was formed. A similar precipitate was formed when a quinine hydrocholoride solution was used in place of the dihydrochloride.

(B) To 50 ml. of 0.0468 *M* sodium dehydrocholate was added dropwise 8.5 ml. of 0.068 *M* quinine hydrochloride; the *p*H of the resulting mixture was 8.4. The solution remained clear initially, but turbidity appeared as addition proceeded, and a colloidal solution was ultimately formed. The turbidity was measured by means of a Klett–Summerson photoelectric colori neter (the zero of which was set for water) and a blue filter (see Table I).

(C) An experiment was carried out as in B but using sodium tauroglycocholate in place of sodium dehydrocholate.

The same concentration (0.0468 *M*) of sodium dehydrocholate and tauroglycocholate was used in experiments B and C, but while the former initially gave a zero reading in the photoelectric colorimeter, the latter solution gave a reading of 104.

Examination of the reaction products showed that, while the colloid formed with sodium dehydrocholate was practically stable toward centrifuging, an appreciable quantity of a coarse sediment was thrown down from that obtained with sodium tauroglycocholate. When quinine dihydrochloride, which is the more acidic, was used instead of quinine monohydrochloride, coarse precipitates began to be formed as soon as the mixture became acidic; this could be prevented by intermittent addition of dilute caustic soda to maintain the reaction alkaline. But, even when the *p*H was controlled in this manner, any further addition of quinine after the molar ratio of quinine to the bile acid in the reaction mixture was about 1:4, resulted in the formation of coarse precipitate.

(D) A 0.0468 *M* quinine monohydrochloride solution was added gradually to a dilute solution of caustic soda until the *p*H of the final mixture was between 8.0 and 9.0. Only a coarse precipitate of the alkaloid was formed.

(E) Equivalent amounts of quinine base and dehydrocholic acid were suspended in water, the *p*H of the solution raised to 8.4 by the gradual addition of caustic soda, and the mixture shaken thoroughly. There was no observable colloid formation.

(F) The reaction mixtures obtained in experiments A, B, C, D, and E were centrifuged for one hour and the supernatant liquids tested for turbidity in the photoelectric colorimeter (Table I).

The colloidal solutions from experiments B and C were then subjected to dialysis in bags of cellophane previously swollen in 64% zinc chloride solution for twenty minutes. The dialysis was carried out against distilled water; the *p*H of the water was subsequently maintained at about 8.4 by the addition of the requisite quantity of caustic soda. Quinine, as well as the bile acid, was found to be diffusing out into the dialysate. The dialysis was continued, with daily changes of water, until no further diffusion of quinine into the outer liquid was noticeable. At this stage, the turbidity readings of the dialyzed liquids were again taken in the photoelectric colorimeter (Table I).

TABLE I

Experiment	Turbidity Reading of Centrifuged Mixture	Turbidity Reading after Exhaustive Dialysis
A	34	Not dialyzed
B	320	2 (did not give the test for quinine)
C	640	108 (there was coarse sediment containing quinine)
D	0	Not dialyzed
E	0	Not dialyzed

The first dialysate was somewhat turbid but subsequent dialysates were clear.

(G) Quinine dehydrocholate was finely powdered, suspended in water, made alkaline with caustic soda and shaken vigorously for fifteen minutes. There was practically no peptization of the solid.

(H) One-tenth gram of quinine dehydrocholate was finely powdered and shaken with 25 ml. of 0.0468 *M* sodium dehydrocholate solution for half an hour and then centrifuged for one hour. A part of the solid was peptized to give a stable colloidal solution. Analysis of this supernatant liquid showed that nearly 10% of the quinine dehydrocholate taken was peptized in this manner.

Use of sodium tauroglycocholate in place of sodium dehydrocholate also resulted in partial peptization of the quinine salt.

(I) The peptized colloidal solution of quinine dehydrocholate solution was also subjected to exhaustive dialysis. A slight turbidity persisted in the liquid even after ten days' dialysis, although tests showed that most of the quinine taken had passed out into the dialysate. Most of the dehydro-

cholic acid was also found to have diffused out through the membrane.

DISCUSSION

It will be seen from the results of the experiments recorded above that quinine readily forms a salt with dehydrocholic acid in chloroform solution, but this salt is sparingly soluble in water and requires more than 1000 parts of water to dissolve it; but when the interaction between a quinine salt and sodium dehydrocholate or tauroglycocholate takes place in aqueous medium, the nature of the product of reaction is found to vary with the conditions of the experiment.

When the quinine salt was added to the bile salt, both being in aqueous solution, and the reaction of the medium was maintained at a *p*H between 8.0 and 9.0 during the addition, the mixture gradually developed turbidity and took the form of a stable colloidal solution (Experiments B and C). The reverse mode of addition, on the other hand, produced a coarse precipitate (Experiment A). It was demonstrated that quinine is precipitated from solution at the *p*H and concentration of the above experiments (Experiment D). Treatment of a mixture of quinine base and dehydrocholic acid (Experiment E) or of quinine dehydrocholate (Experiment G) with dilute caustic soda solution produced no observable effect. Quinine dehydrocholate was, on the other hand, found to be peptized by excess of an alkaline solution of sodium dehydrocholate (Experiment H), although the proportion of the solid peptized in this manner was found to be about 10%.

Dialysis Experiments F and I on the colloids obtained from Experiments B, C, and H show that, in all these cases, the quinine as well as the bile acid diffused out through the cellophane membrane, to a greater or less extent. The diffusion of quinine was complete in the case of the colloid obtained from Experiment B but incomplete with the colloids from Experiments C and H. It thus appears that bile salts, under favorable conditions, can "solubilize" quinine in an alkaline medium.

Quinine, when taken orally, is considered to be absorbed mainly from the intestines (16). Bile is also normally present in this region. When *ingested quinine* enters the intestine in the form of a solution and reacts with the bile salts, the result, presumably, would be a colloidal solution as in Experiment C. Since quinine is found to diffuse through swelled cellophane membrane from this type of colloid, it might also be expected to penetrate the membranes of the cells lining the stomach and thus enter the circulatory system.

It will be seen from Experiment C that a considerable excess of bile salts would be necessary in order to have the quinine in the form of a stable colloidal solution. It would therefore follow that the presence of an adequate quantity of bile salts would be necessary for the efficient absorption of quinine from the intestine. Recent work by Fredericks and Hoffbauer (17) has shown that attacks of therapeutic malaria are almost always accompanied by hepatic disturbances; these may disturb the flow of the normal quantity of bile from the liver into the intestine. In the treatment of malaria by the oral administration of quinine, therefore, the simultaneous administration of an effective choleretic like dehydrocholic acid is likely to help in the absorption of quinine and thereby to enhance its therapeutic effect, particularly in those cases where deficiency of bile secretion is suspected.

Further, Experiment H indicates that the administration of quinine in the form of salts of bile acids is not likely to be of any special advantage. Such a salt of quinine would be peptized or solubilized by bile salts only partially, the major part remaining as an insoluble precipitate, the absorption of which would not be very efficient.

SUMMARY

1. Quinine and dehydrocholic acid react in chloroform solution to form a salt containing one molecule of each component. This salt is sparingly soluble in water.

2. When added to an alkaline bile salt or dehydrocholate solution, a quinine salt in aqueous solution forms a colloidal solution. On dialysis of this colloidal solution, both quinine *and* the bile acids diffuse out through the cellophane membrane.

It has been suggested that this solubilizing action of bile salts on quinine in alkaline medium provides a mechanism for the absorption of quinine from the intestine.

3. The simultaneous administration of an effective choleretic, preferably dehydrocholic acid, in the quinine therapy of malaria might augment the therapeutic efficacy of the drug.

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A Spectrophotometric Method of Analysis of Cascara Sagrada Preparations*

By MELVIN R. GIBSON and ARTHUR E. SCHWARTING

A method of quantitative analysis of Cascara Sagrada preparations dependent upon the isolation of the hydroxy-methyl-anthraquinones on a chromatographic column of magnesium oxide and celite was devised. The anthraquinones were quantitatively eluted from the column and the concentration of the resulting solutions determined from the results of spectrophotometric examinations. Calculations of concentration of total anthraquinones were made on the basis of a determined extinction coefficient for pure emodin. The results of the analyses of fluidextracts by this method gave results correlating quite closely those previously reported in the literature for a different process.

THE PROBLEM of obtaining an accurate means of quantitative analysis for the anthraquinone-bearing drugs has been under investigation for over a half century. The methods which have received attention during this time include (*a*) gravimetric extraction, (*b*) gravimetric precipitation, and (*c*) colorimetric. Gravimetric extractions have been studied by Aweng (1), Daels (2), Tunmann (3), Fuller (4), and Hebeisen (5). Gravimetric precipitation methods have been investigated by Tschirch and Edner (6), and Tschirch and Pool (7). Colorimetric methods have been suggested by Tschirch and Heipe (8), Tschirch (9), Bromberger (10), Warin (11), Maurin (12), and Fuller (13).

In 1926 Tumminkatti and Beal (14) proposed a method of analysis involving Daels' method with modifications to include quantitative adsorption of the anthraquinones on Norit decolorizing carbon. This process involved the weighing of the impure extracted anthraquinones before and after treatment with Norit and calculating the anthraquinone quantity to be the difference.

The results of this method of assay seemed to the authors to be indicative that this method was the best that has been evolved in solving the problem of quantitative analysis of anthraquinone-bearing drugs. However, in the process of an extensive study of the chromatographic isolation and spectrophotometric analysis of the anthraquinone constituents of the bark of Cascara Sagrada being carried on by the authors, a simplification of this process, following generally the same line of reasoning, was believed possible.

One of the disadvantages of the process of Tumminkatti and Beal is the tedious shaking-out process in the isolation which yields large volumes of liquids that become difficult to handle and which must eventually be concentrated. To alleviate this difficulty as well as the weighings of minute quantities, chromatographic isolation and quantitative spectrophotometry are suggested.

Ernst and Weiner, as discussed by Zechmeister and Cholnoky (15), were the first to study extensively the anthraquinone-bearing drugs by means of chromatography. The resolution of an alcoholic extract into anthraquinone and anthranol layers was accomplished for ten anthraquinone-bearing drugs.

EXPERIMENTAL

In the method to follow, the isolation of the anthraquinones (the term *anthraquinones* is used in this paper to mean the trihydroxy-methyl anthraquinones of the emodin series) is dependent upon chromatography and the quantitative determination upon the adsorption of light as measured by the spectrophotometer. The method of extraction of anthraquinones present in the "free" or uncombined state and those present in the "combined" or as aglycones of glycosides was essentially that of Daels (2). For the analysis of the powdered extract and powdered bark of Cascara Sagrada, 5 Gm. was used and for the fluidextracts of Cascara Sagrada, 5 cc. was pipetted onto shredded filter paper and allowed to dry in a 56° constant temperature oven. The powder or dried shreds were then placed in a 500-cc. round bottom flask with 250 cc. of chloroform and refluxed for one hour on a steam bath. The

* Received February 13, 1947, from the Department of Pharmacognosy, College of Pharmacy, University of Nebraska, Lincoln, Nebr.

chloroform was decanted and the Cascara washed with two 10-cc. portions of chloroform. This chloroform solution contains the "free" anthraquinones. The partially extracted material was then refluxed in the same flask for two and one-half hours in the presence of 250 cc. of chloroform and 50 cc. of 25% sulfuric acid. The chloroform and aqueous mixture was decanted from the marc which was then washed with three 20-cc. portions of chloroform. The chloroform was separated from the aqueous layer in a separatory funnel. This chloroform solution contains the anthraquinones present in the preparations in glycosidal form which have been separated by hydrolysis. This solution will be referred to as the "combined" anthraquinones.

These solutions were then allowed to pass by continuous gravity-flow through chromatographic tubes packed with a mixture of Westvaco Adsorptive Magnesia #2641 (1 part) and celite (2 parts). The chloroform solution of free anthraquinones yielded a small bright red layer of anthraquinones at the top of the column followed by a yellow layer of anthranols which gradually became dispersed throughout the column. The anthraquinones remained in a definite red layer at the top. The solution passing through the column did not yield a Borntraeger Reaction (16) indicating the absence of anthraquinones. Sectioning of the extruded column proved that all anthraquinones were adsorbed in the top red layer. This would be expected since the basicity of the magnesia column causes the bright red layer to form wherever anthraquinones are present. The red layers formed by the chromatograms of free anthraquinones were 5 to 7 ml. wide and those for combined anthraquinones were from 12 to 24 ml. wide in standard chromatographic tubes 19 ml. in diameter.

The columns were extruded and the red layers cut from the remainder of the tube. Ordinary methods of eluting the adsorbed anthraquinones were unsuccessful. However, by treating the dry red powder with 10% hydrochloric acid the chemical reaction of the acid with magnesium oxide released the adsorbed anthraquinones which were readily shaken from the acid-aqueous mixture, in which they are insoluble, with successive portions of chloroform. For the free anthraquinone layer obtained from the fluidextracts, chloroform in 25-cc. portions was shaken with the mixture until the chloroform washings, measured in a volumetric flask, totaled 250 cc. For the powdered extract a total of 500 cc. of chloroform was shaken with the mixture and for the powdered bark a total of 1000 cc. was used. Tests of subsequent shakings beyond these volumes demonstrated an absence of anthraquinones. The procedure for the elution of the layers containing the combined anthraquinones of all samples was similar to the method used to elute the free anthraquinones. The chloroform washings, however, were continued until the solutions in each case measured 1000 cc. in a volumetric flask. Before spectrophotometric tests could be made 10 cc. of the chloroform solution representing the free anthraquinones of the powdered drug and the chloroform solutions of the combined anthraquinones of the powdered extract and the powdered drug were each diluted to 100 cc. quantitatively. All other solutions were examined as originally prepared.

These chloroform solutions were examined spectrophotometrically using a Coleman Universal spectrophotometer in the visible region of the spectrum at ten-millimicron intervals using the null method. The solutions were in cuvettes 1.3 cm in diameter using chloroform as the standard. A complete analysis of the eluted free and combined chloroform solutions of the respective chromatograms of sample C-2, a powdered extract, is shown in Table I.

Pure emodin was prepared from Cascara Sagrada bark by the Jowett (17) method and a solution of this examined spectrophotometrically gave results as shown in Table I. Preliminary investigations of the other anthraquinones isolated from Cascara indicate maximum light absorption in the 400 to 440 mμ range. The maximum absorption of the red layers is the same as that for pure anthraquinones indicating anthraquinone isolation in the red layer of the chromatogram.

TABLE I.—COMPARATIVE TRANSMITTANCE OF ELUTED CHROMATOGRAPHIC LAYERS AND EMODIN

Wave Length, mμ	C-2 Free	C-2 Comb.	Emodin
400	54.5	66.0	69.5
410	57.0	68.5	67.0
420	58.0	69.0	64.0
430	58.0	69.5	60.5
440	58.5	70.0	59.0
450	60.5	71.5	59.5
460	64.5	74.3	63.0
470	71.5	79.0	68.7
480	79.0	84.5	76.5
490	86.0	88.5	83.0
500	91.0	93.0	88.5
510	94.0	94.0	91.5
520	96.5	96.5	94.0
530	97.5	98.0	94.0
540	98.0	98.0	95.0
550	99.5	98.5	96.0
560	99.5	98.5	96.0
570	99.5	98.5	96.5
580	99.5	99.0	96.5
590	99.5	99.0	96.5
600	99.5	99.0	97.0

Since emodin is the anthraquinone present in the largest quantity, all quantitative calculations are made on the emodin basis at the wave length of maximum absorption for emodin, 440 mμ. Since all anthraquinones show maximum or near-maximum absorption at this wave length and are simply position isomers of emodin the molecular extinction coefficient (K) for emodin is a workable basis.

Emodin was proved to conform with Beer's law in that the absorptance of the solution is directly proportional to the concentration of the solute (number of absorbing molecules of absorbing sub-

TABLE II.—PERCENTAGE TRANSMITTANCE AT 440 mμ

Sample	Free	Combined
A-1	74.0	36.0
A-2	76.0	35.3
A-3	35.3	45.0
B-1	58.0	17.0
B-2	72.0	17.0
B-3	45.0	17.0
C-1	60.0	69.0
C-2	58.5	70.0
C-3	64.5	68.3
D-1	77.0	53.0
D-2	77.0	52.0
D-3	77.0	52.7

TABLE III.—CALCULATED WEIGHTS (GM./5 CC. OR GM./5 GM.) OF ANTHRAQUINONES

Sample	Free	Combined	Total
A-1	0.0010	0.0135	0.0145
A-2	0.0009	0.0137	0.0146
A-3	0.0034	0.0105	0.0139
B-1	0.0017	0.0232	0.0249
B-2	0.0010	0.0232	0.0242
B-3	0.0026	0.0232	0.0258
C-1	0.0033	0.0490	0.0523
C-2	0.0035	0.0470	0.0505
C-3	0.0028	0.0503	0.0531
D-1	0.0317	0.0836	0.1153
D-2	0.0317	0.0869	0.1186
D-3	0.0317	0.0843	0.1160

stance). Three solutions of known concentration of emodin were prepared. Solution No. 1 contained 0.010 mg. per cc., No. 2, 0.005 mg. per cc., and No. 3, 0.0025 mg. per cc. At 440 mμ, No. 1 gave 47.0% transmittance, No. 2, 68.5%, and No. 3, 84.7%. When the transmittance is plotted on a logarithmic axis against concentration, a straight line results indicating conformity to Beer's law.

Using the formula $C = \frac{\log_{10} Io/I}{Kb}$ the emodin K is 6830. In the formula, C equals the concentration in moles per liter, $\log_{10} Io/I$ is the logarithm of the ratio of incident to emergent light, K is the molecular extinction coefficient, and b is the thickness of the absorption cell. The value of the molecular extinction coefficient for emodin was used in conjunction with this formula in subsequent calculations of the anthraquinone content of the Cascara preparations.

Using the processes of extraction, isolation, and quantitative measurement as indicated above, the following preparations were analyzed. Samples of the A series represent a U. S. P. Fluidextract of Cascara Sagrada made by a prominent manufacturer; samples of the B series represent a U. S. P. Fluidextract of Cascara Sagrada by a different manufacturer; samples of the C series represent the powdered U. S. P. Extract of Cascara Sagrada made by the same manufacturer as the Fluidextract A; and samples of the D series represent the bark of Cascara Sagrada in a powder finer than #40.

The percentage transmittance of the anthraquinone 440 mμ is given in Table II. Table III gives the calculated results in grams.

SUMMARY

From these results several conclusions may be drawn concerning the process.

1. As exemplified by samples A the results for free and combined anthraquinones vary only a few milligrams from the results reported by Tumminkatti and Beal in their process using an extract from the same manufacturer.

2. As shown by the results of sample B there is considerable difference in anthraquinone content of the products of different manufacturers. This was also found to be true by Tumminkatti and Beal.

3. The processes of extraction and isolation seem to be consistently efficient as several successive samples gave exactly the same spectrophotometric readings and all were within 1 or 2 mg.

4. The preparations of Cascara Sagrada represent only a fraction of the anthraquinones present in the bark.

An efficient method is proposed for the quantitative analysis of Cascara Sagrada preparations aimed at eliminating some of the factors influencing variable results of previous methods of analysis.

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The Preparation and Properties of the Perchlorates of Some Choline Esters*,†

By FREDERICK K. BELL and C. JELLEFF CARR

The preparation and properties of acetyl, benzoyl, furoyl, and thenoyl choline perchlorates are described. It is shown that perchloric acid may be used to isolate choline esters from their reaction mixtures.

THE PREPARATION of a number of choline esters which were desired for pharmacologic study in this laboratory was greatly facilitated by the observation that acetyl choline can be readily obtained in a well-defined crystalline form as the perchlorate salt. Furthermore, even casual observation revealed that this salt showed considerable stability toward atmospheric moisture in marked contrast to the well-known behavior of the chlorides of most of the choline esters that have been prepared. Our hope that the desired choline esters would also yield similar perchlorate salts was fulfilled.

A survey of the literature reveals that little attention has been given to the preparation of the perchlorate salts of choline esters. Hofmann and Hobold (1) have prepared and described in detail choline perchlorate and the nitrate ester of choline perchlorate. In accord with their recommendations, the latter compound has been found very satisfactory for the identification of choline (2) and its pharmacology has been investigated in this laboratory (3). Binet and Burstein (4) studied the action of propionylcholine perchlorate on the bronchomotor apparatus but give very little information concerning the chemistry of the salt.

It is the purpose of this communication to describe the preparation and properties of the perchlorates of four choline esters, *viz.*, acetyl, benzoyl, furoyl, and thenoyl. The latter two compounds are new esters that have not been previously prepared. It will be noted that if the sulfur atom in the thiophene ring of the thenoyl compound is replaced by oxygen then the furoyl ester is obtained.

EXPERIMENTAL

Acetyl Choline Perchlorate.—This salt can be readily obtained in the following manner and in good yield. To a 10% solution of acetyl choline chloride or bromide in absolute ethyl alcohol, add dropwise, with stirring, 60% perchloric acid solution until slightly more than the calculated amount of acid has been added. Chill the mixture in ice while stirring. The perchlorate is precipitated as a mass of feathery white crystals, which can be readily removed by filtration. If the crystals are washed with a little ice-cold absolute alcohol and then with dry ether, a pure product is obtained. After 2 recrystallizations from absolute alcohol the compound melted at 116–117°.[1] The crystals occur as white rods or needles, are double refracting, and are readily soluble in water, acetone, and hot absolute alcohol. Microanalysis in duplicate gave the following results:

Anal.—Calcd. for $C_7H_{16}O_6NCl$: C, 34.22%; H, 6.57%; N, 5.70%. Found: C, 34.45% and 34.32%; H, 6.90% and 6.82%; N, 5.58% and 5.59%.

The benzoyl, furoyl, and thenoyl esters of choline were readily prepared by condensing choline chloride with the corresponding acid chloride. Thenoyl chloride was prepared by treating α-thenoic acid (kindly supplied by the Socony-Vacuum Company) with thionyl chloride (5).

In the case of each of the 3 esters the procedure was similar. In a suitable flask equipped with a vertical air condenser, choline chloride was treated with the acid chloride in the proportion of 1 mole of the former to 2 or 3 moles of the latter. The mixture was heated on a steam bath for several hours. The mixture was cooled and then extracted several times with dry ether to remove the excess acid chloride and hydrochloric acid. The residue, consisting of the chloride of the ester, was dissolved readily in absolute alcohol. On treating this solution with a slight excess of 60% perchloric acid solution the perchlorate salt was precipitated and subsequently purified by recrystallization.

Each of the 3 salts appears to be completely nonhygroscopic. A report on the pharmacology of these compounds is in the course of preparation.

Benzoyl Choline Perchlorate.—White double refracting crystals, m. p. 203–205°; readily soluble in acetone, much less soluble in hot absolute alcohol, sparingly soluble in water.

* Received March 10, 1947, from the Department of Pharmacology, School of Medicine, University of Maryland, Baltimore, Md.

† The expense of this study was defrayed in part by a grant from the Board of Trustees of the United States Pharmacopeial Convention.

[1] All melting points reported were determined with the Fisher-Johns melting-point apparatus.

Anal.—Calcd. for $C_{12}H_{18}O_6NCl$: C, 46.83%; H, 5.90%; N, 4.55%. Found: C, 47.16% and 47.45%; H, 6.22% and 6.11%; N, 4.50% and 4.52%.

Furoyl Choline Perchlorate.—White double refracting crystals, m. p. 143–144°; readily soluble in acetone, soluble in water, and less soluble in absolute alcohol.

Anal.—Calcd. for $C_{10}H_{16}O_7NCl$: C, 40.34%; H, 5.42%; N, 4.71%. Found: C, 40.37% and 40.29%; H, 5.78% and 5.40%; N, 4.76% and 4.60%.

Thenoyl Choline Perchlorate.—White double refracting scales or plates; m. p. 189–190°; readily soluble in acetone, sparingly in alcohol and water.

Anal.—Calcd. for $C_{10}H_{16}O_6NHClS$: C, 38.28%; H, 5.14%; N, 4.46%. Found: C, 38.50% and 38.61%; H, 5.26% and 5.60%; N, 4.48% and 4.49%.

As has been previously noted, with the first preparation of acetyl choline perchlorate we were impressed by the apparent stability of this compound toward atmospheric moisture. This is in definite contrast to the behavior of the chloride which is usually dispensed in sealed glass ampuls because of its marked hygroscopicity. Acetyl choline bromide is regarded as more stable than the chloride in this respect. We have carried out several simplified experiments to demonstrate the comparative behavior of these 3 salts toward atmospheric moisture under controlled conditions.

Four similar weighing bottles were selected and each of 3 of these was weighed against the 4th bottle as a tare. Into 1 bottle was introduced approximately 0.1 Gm. of acetyl choline perchlorate which had been stored over calcium chloride. The bottle was immediately closed and then weighed. In the same manner a 0.1-Gm. sample of acetyl choline bromide was introduced into a second weighing bottle and weighed. This salt was removed from a new and unopened bottle of the Eastman Kodak product. The label was removed from a sealed glass ampul containing 0.1 Gm. of Merck's acetyl choline chloride and the ampul was then cleaned and dried. The ampul was opened and immediately placed in the third weighing bottle, which was promptly stoppered and then weighed.

A water-sulfuric acid mixture adjusted to give a relative humidity of 50% at a room temperature of 25° was introduced into a desiccator in place of the usual desiccant. The weighing bottles were placed in the desiccator and opened, after which the cover was placed on the desiccator. At definite intervals the bottles were stoppered, removed from the desiccator, and weighed, after which they were returned to the desiccator for further exposure to the humidified atmosphere. The weighing bottle used as a tare was subjected to the same treatment throughout the experiment.

A typical series of weighings is shown in Table I. These results indicate that the perchlorate is completely stable under the conditions of the experiment. On the other hand the chloride takes up moisture rapidly and the bromide shows a definite hygroscopicity which, however, is considerably less than that of the chloride.

In the light of these results we subjected the bromide and the perchlorate to a more drastic test. The sulfuric acid mixture in the desiccator was replaced with water and under these conditions a relative humidity of 100% was to be expected. Samples of a tenth of a gram of each of the 2 salts were weighed out and, after exposure to this atmosphere for one hour, were weighed again. The bromide had visibly liquefied and showed a weight increase of 10 mg. or 10%. No significant change in weight of the perchlorate sample was observed.

We may therefore conclude that the perchlorate is completely nonhygroscopic. It is readily soluble in water and hence represents a very convenient form of acetyl choline for the pharmacologist, especially in those cases where this substance must be weighed out in small quantities with analytical precision.

TABLE I

Total Time of Exposure, Hr.	Total Change in Weight of 0.1-Gm. Samples, Mg.		
	Chloride	Bromide	Perchlorate
1/4	1.7	0.7	−0.3
1/2	2.8	1.2	−0.2
1	3.9	1.5	−0 1
1 1/2	5.4	1.8	+0.1
2 1/2	7.8	2.4	−0.1
3 1/2	10.6	3.4	0.0
4 1/2	12.7	4.0	−0.1
10 1/2	20.6	6.1	+0.1
51 1/2	39.2	8.1	−0.2

SUMMARY

1. The preparation and properties of acetyl, benzoyl, furoyl, and thenoyl choline perchlorates have been described.

2. The results indicate that perchloric acid may be used to considerable advantage to isolate choline esters from their reaction mixtures since in general the perchlorate salts are well-defined crystalline and nonhygroscopic substances.

3. Acetyl choline perchlorate, which can be readily prepared, is nonhygroscopic and readily soluble in water. It therefore represents a highly desirable form of acetyl choline particularly for the pharmacologist's use.

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Organic Ozonides as Chemotherapeutic Agents. I. Chemical Studies*

By GEORG CRONHEIM†

Chemical and physical properties of ozonized olive oil have been investigated. Ozonized olive oil decomposes in the presence of water into medium length mono- and dibasic acids and aldehydes and hydrogen peroxide. Organic peroxidic compounds are formed as intermediates. It is suggested that the beneficial clinical results which have been obtained with ozonized olive oil are due to the germicidal properties of the peroxides and to specific actions of the final decomposition products.

THE PHARMACEUTICAL use of oxygen releasing substances dates back as far as 1818 when Thenard discovered hydrogen peroxide. The compounds employed since then were and still are almost exclusively inorganic or organic peroxides. Very little attention has been paid to ozonides many of which decompose with the release of nascent oxygen and can thus be used in the same manner as peroxides.

Although Schoenbein was the first to observe in 1855 the addition of ozone to the ethylene linkage, it was Harries who from 1901–1916 studied in detail the formation and the physical and chemical properties of organic ozonides. In 1914 Knox (1) filed a patent application for "a medicinal compound comprising the ozonide of the triglyceride of a hydroxylated fatty acid of the $C_nH_{2n-2}O_3$ series." This ricinoleic acid ozonide was supposed to be an "improved, efficient and emollient laxative" with germicidal action upon pathogenic microorganisms in the intestines.

As a matter of curiosity mention should also be made of the book *Experiments with Oxygen on Disease*, published in 1921 by James Todd (2). The author first deals in detail with the manufacture of ozonized olive and cod-liver oils. He then goes on to describe how he obtained "miraculous cures" with oral doses of "from 100 to 300 drops daily of the ozonized oil alone or with a small quantity of whisky" in Bright's disease, tuberculosis, etc.

A scientific investigation of the germicidal properties of ozonides in form of ozonized olive oil was started through the investigations of Harada (3) and Stevens (4). The first clinical report was published by Bender and Blanchard (5) in 1938, followed by reports from Sawyer (6), Sackin (7), Leonard and Engle (8), Greenberger and Helfert (9), Barrows (10), and recently by Sharlit (11).

In all these cases the ozonide was used topically. The only investigation on the internal use of ozonides seems to be that of Butz and La Lande (12) who studied the anthelmintic action of several ozonized oleic acid esters in dogs infested with ascarides.

The reason that ozonized olive oil has been used in these investigations is due to the fact that olive oil with its relatively high concentration of olefinic double bonds is a very convenient starting material. Furthermore, as will be shown later, it has certain advantages regarding stability and odor over other investigated materials and synthetic oleic acid esters.

Ozonized olive oil[1] is obtained by forcing an ozone-air mixture through olive oil at room temperature. Under proper conditions, the ozone will react quantitatively with the double bonds in the unsaturated fatty acids forming the corresponding ozonides. It has been found that a partly (50 per cent) ozonized olive oil represents the most practical form for pharmaceutical application and such a product was used for the most part in the present investigation.

The main components in olive oil with olefinic double bonds are oleic and linoleic acids. Calculated as triolein and trilinolein, they comprise from 75–85 per cent and from 6–10 per cent, respectively, of the olive oil.

* Received Feb. 10, 1947, from the Research Laboratories of The G. F. Harvey Company, Saratoga Springs, N. Y.
† Present address: The S. E. Massengill Company, Bristol, Tenn.

[1] This product is available under the trade name "Olzo" from The G. F. Harvey Company, Saratoga Springs, N. Y.

the rest consisting of the glycerides of saturated fatty acids.[2] A partly ozonized olive oil can thus be best described as a solution of triolein ozonide in olive oil, if one disregards the small amount of trilinolein ozonide which may be present. In this and the following paper the product will be designated simply as ozonized olive oil.

It shows that the decomposition can proceed in two ways, and that water plays an essential role in one of them. The final compounds are acids, aldehydes, organic peroxides, and hydrogen peroxide. Quantitative data for the rate of decomposition of ozonized olive oil are given in Tables I and II.

TABLE I.—SPONTANEOUS DECOMPOSITION OF OZONIZED OLIVE OIL

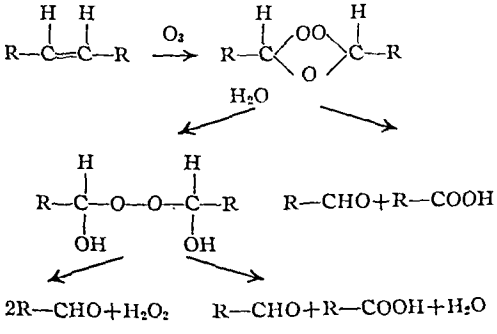
Sample Kept at	Active Oxygen in % (w/w) Untreated 2°	Untreated 22°	Dried 22°	Free Acids in Millimol./Gm. Untreated 2°	Untreated 22°	Dried 22°
0 wk.	1.37	1.37	1.37	0.71	0.71	0.71
6 wk.	1.32	1.21	1.22	....	...	....
13 wk.	1.29	1.04	1.18	1.07	1.16	1.36
21 wk.	1.27	0.92	1.10	....	..	....
31 wk.	1.24	0.79	1.06	1.44	1.92	2.02

TABLE II.—AMOUNT OF ACTIVE OXYGEN IN OZONIZED OLIVE OIL AND OF HYDROGEN PEROXIDE IN THE AQUEOUS PHASE AFTER 24 HOURS' CONTACT AT 22°

Sample No.	Active Oxygen in % (w/w) 1	2	3	Hydrogen Peroxide, Mg % 1	3
Original Concentration	0.77	0.80	1.41	..	..
After Contact with:					
Distilled water	0.82	0.90	1.49	10.2	11.2
0.1 *M* $NaHCO_3$	0.71	0.89	1.33	4.6	6.5
0.1 *M* NaH_2PO_4	0.81	0.84	1.55	9.8	9.9
Ringer solution	..	0.79	.	..	.

Ozonized olive oil is an almost colorless oil which starts to solidify at a temperature, below 10°. It is very soluble in chloroform, ether, and petroleum ether, and, unlike olive oil, fairly soluble in alcohol and acetone. The surface tension of ozonized olive oil is quite low, approximately 30 dynes/cm.

The structural formula and the decomposition of aliphatic ozonides has been the subject of numerous investigations and controversies, a detailed summary of which is given by Long (13). In a greatly simplified form it can be schematically written as follows:



[2] Other unsaturated compounds, like arachidonic acid, are present only in traces.

EXPERIMENTAL

Due to the complexity of the decomposition reaction no simple analytical procedure for the evaluation of ozonized olive oil is known. For practical purposes an iodometic procedure has been adopted with the results expressed in per cent by weight of active oxygen.

An amount of from 0.1 to 0.2 Gm. of ozonized olive oil is weighed in a little dish, transferred to a 250-ml. Erlenmeyer flask, and dissolved in 10 ml. of chloroform. To this solution is added a mixture of 38 ml. of glacial acetic acid and 2 ml. of a 50% aqueous potassium iodide solution. The flask is closed with a glass stopper and kept with occasional shaking for exactly forty-five minutes in a dark place. After this time, 75 ml. of water are added and the liberated iodine is determined by titration with 0.02 *N* sodium thiosulfate solution (1 ml. = 0.16 mg. O.)

If this method is followed exactly, it will give reproducible results. If one calculates the concentration of the ozonide on the basis that each mole of active oxygen is equivalent to one mole of ozonide one finds only about 70% to 80% of the ozonides present. The reason for this deficiency is partly due to the fact that the reaction has not reached the end point after forty-five minutes but proceeds very slowly for another sixty to ninety minutes. More important, however, is the fact that in the presence of glacial acetic acid part of the ozonide will be split

directly into an aldehyde and an acid without liberation of iodine.

Ozonized olive oil will slowly decompose due to the inherent lability of the triolein ozonide molecule. This decomposition cannot be prevented completely but it can be retarded by removing all moisture from the finished product and storing it at low temperature. An example is given in Table I which contains also values for the amounts of free acids present under the different conditions.

It is interesting to note that the formation of free acids is relatively little influenced by the storage temperature and is not affected by the moisture present.

Since ozonized olive oil is intended only for topical application where moisture is always present, the decomposition in this case is of particular interest and was studied in the following manner:

Ten milliliters of distilled water or 0.1 *M* solution of sodium bicarbonate or monosodium phosphate (*p*H 8.2 and 4.5, respectively) in a small (25-ml.) beaker were completely covered with ozonized olive oil (approximately 0.4 Gm.). The samples were then kept at 37° for twenty-four hours. After this time, the amount of available active oxygen in the oil was determined in the above described manner and also the amount of hydrogen peroxide in the aqueous phase. The average results of a number of experiments are summarized in Table II.

At first it seemed somewhat paradoxical that, after prolonged contact with water, the ozonized oil should yield more active oxygen than it showed originally. The explanation is to be found in the different ways in which the ozonides can be decomposed. It had been indicated that glacial acetic acid can split triolein ozonide directly into an aldehyde and an acid without the liberation of active oxygen in any form. This means that, in the standard procedure of analysis, a certain amount of the ozonide will not react with hydriodic acid and that, therefore, the obtained results are too low. If, however, the oil has been in contact with water for a prolonged period of time, part of it has already been decomposed with the formation of per-compounds. If the standard method of analysis is then applied to this already partly decomposed product, the per-compounds will hydrolyze quantitatively while the remaining ozonides will decompose to the same extent as before. Thus, a simple calculation of the reaction equilibrium proves that a larger amount of active oxygen should be found if the oil was in contact with water before the analysis was made.

Table II contains also values for the amount of active oxygen in the aqueous phase expressed in terms of hydrogen peroxide. As can be expected, the decomposition of both the oil and hydrogen peroxide proceeds at a somewhat faster rate if the *p*H of the solution is shifted to the alkaline side.

The above explanation, that ozonized olive oil after prolonged contact with water shows an apparently greater concentration of active oxygen than the original sample, is also supported by another observation. The kinetics of the reaction employed in the analytical procedure indicate that it is neither of the first nor of the second order. This means that the results are affected by some factors other than the concentration of either ozonide or water. The glacial acetic acid and hydriodic acid are present in such excess that their influence upon the rate of the reaction can be neglected. One must, therefore, conclude that several reactions proceed at the same time.

The experiments which are summarized in Table II give some information as to the rate of decomposition of ozonized olive oil in contact with water and at different *p*H. There are, however, several other factors which, in the practical application, might influence this decomposition. Since the chemical structure of the ozonized oil still resembles that of fats, the possibility of an enzymatic cleavage was of importance. Furthermore, since the product is recommended as a wound dressing, the question arose whether or not surface active forces on cell membranes and tissue surfaces might influence the reaction.

Table III contains a summary of experiments similar to those described above where the water was replaced by hemolyzed blood, coagulated blood, serum, or minced muscle tissue from healthy rats. Of these substances, coagulated blood and serum do not alter appreciably the amount of available active oxygen remaining in the oil while hemolyzed blood, and especially muscle tissue, decrease this amount clearly. This becomes still more evident if one considers that the *p*H in both cases is slightly below 7 and the most comparable figures are those obtained with distilled water where the detectable amount of active oxygen had shown an increase. The results thus indicate clearly that under these conditions the decomposition of ozonized olive oil is actually accelerated by contact with either hemolyzed blood or muscle tissue.

TABLE III.—AMOUNT OF ACTIVE OXYGEN IN % (*w/w*) IN OZONIZED OLIVE OIL AFTER 24 HOURS CONTACT WITH BIOLOGICAL MATERIAL

Sample No.	1	2
Original Concentration	0.77	0.81
After Contact with:		
Distilled water	0.82	0.90
Hemolyzed blood	....	0.72
Coagulated blood	....	0.81
Serum	....	0.78
Muscle tissue	0.63	....

Since it is possible that the final products of the decomposition of ozonized olive oil, other than nascent oxygen or hydrogen peroxide, might have some pharmacological action of their own, they shall be mentioned briefly. The following list contains under 1–4 the 2 aldehydes and 2 acids which will be formed upon the decomposition of triolein ozonide. Since olive oil contains up to 10% linoleic acid which

is also able to form an ozonide, the corresponding decomposition products are included under numbers 5–9. In this tabulation, the possible existence of glycerylesters has been disregarded.

1. Pelargonaldehyde
2. Pelargonic acid
3. ω-Formylcaprylic acid
4. Azelaic acid
5. Caproaldehyde
6. Caproic acid
7. Malonaldehyde
8. Formylacetic acid
9. Malonic acid

TABLE IV.—STABILITY OF VARIOUS OZONIZED OILS

Ozonide of	Initial Concentration in % (w/w)	Active Oxygen In % of Initial Concentration after 8 Wk. at 2°	22°	35°	Free Acids in Millimole./Gm. Freshly Ozonized	After 8 Wk. at 22°
Olive oil	3.52	92	82	60	0.71	1.17
Corn oil	4.78	88	77	48	.	. .
Peanut oil	7.53	100	92	44	2.20	3.73
Peanut oil	3.99	100	95	60	1.13	2.35
Persic oil	4.24	90	72	46	0.74	1.09
Triolein	6.86	91	80	58	....	.
Ethyl oleate	9.71	95	85	46	9.02	13.34
Ethyl oleate	3.78	95	88	58	3.75	3.92

All these compounds have been found actually as decomposition products of oleic acid ozonide and linoleic acid ozonide by various investigators (14). Compounds 1, 2, 5, and 6 belong to the group of saturated fatty acids and their aldehydes while the others are dibasic acids and their aldehydes. This distinction is important because these 2 groups have quite different biochemical and physiological properties.

Kisch (15) studied the influence of the sodium salts of low molecular fatty acids on cell metabolism of different tissues, and found that they provoke a slight increase. However, his experiments went only as far as oenanthic acid. D'Alessandro and Petrucci (16) found that several fatty acids, among them pelargonic acid, will increase considerably the glycolytic ability of red blood cells. Mention should also be made of the observation by Carruthers concerning the inhibiting action of heptyl aldehyde on carcinogenic substances (17).

There are several publications with regard to specific actions of dibasic acids. J. H. Mueller (18) was able to isolate from liver extract a substance which stimulated the growth of diphtheria bacilli in concentrations as low as 1 part in 200 million. He could identify this substance as pimelic acid which is a lower homolog of azelaic acid. He also tested azelaic acid and other simple dibasic acids, but found none effective. English, Bonner, and Haagen-Smit (19) in their study of the so-called wound hormones of plants found that saturated dibasic acids with 8 or more carbon acids, which include azelaic acid, are able to induce renewed growth in mature plant cells.[3]

[3] These substances should not be confused with plant growth substances which only accelerate the normal development and growth.

DISCUSSION

All these observations have one fact in common, namely, that certain saturated mono- and dibasic aliphatic acids can have a stimulating effect on living cells and several such acids are formed when ozonized olive oil is hydrolyzed in the presence of water.

The implications to the present study are obvious. Several clinical and laboratory observations have indicated that ozonized olive oil promotes the normal wound healing process. The above-mentioned investigations could give one possible explanation of this observed action. In addition, ozonized olive oil also has considerable germicidal activity which will be described in a subsequent paper.

The fact, that the decomposition products of ozonized olive oil are acids or of an acidic character, should not prove objectionable to the use of this compound in wounds. It is known that the wound secretion must be acid before the proper healing process can take place (20), because an acid reaction offers better resistance to invading organisms. Some investigators even believe acidity acts as a stimulus to formation of primary mesoblastic tissue and a growth of granulation and accelerated wound healing (21).

In addition to olive oil several tests were made with other vegetable oils (corn oil, peanut oil, persic oil) as well as with synthetic products (ethyloleate and triolein). The formation of the ozonides proceeded equally well in all cases. There are some variations in the stability but they are not significant (Table IV). Of some importance, however, is the fact that the disagreeable odor which the ozonides of vegetable oils develop in time was least offending in olive oil. The

ethyloleate ozonide was quite different. The odor is rather inconspicuous and the product is noticeably less viscous than the other investigated ozonides.

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Organic Ozonides as Chemotherapeutic Agents. II. Antiseptic Properties*

By GEORG CRONHEIM†‡

The germicidal properties of triolein ozonide in the form of ozonized olive oil have been investigated. Ozonized olive oil *in vitro* is bactericidal to *Staphylococcus aureus, Staphylococcus albus,* and *Streptococcus hemolyticus* and fungicidal to *Trichophyton mentagrophytes, Trichophyton purpureum, Microsporon audouini,* and *Microsporon lanosum.* Against *Monilia albicans* it is only fungistatic.

IN A PREVIOUS PAPER (1) it had been shown that ozonized olive oil decomposes in the presence of water into peroxidic compounds including hydrogen peroxide as well as aliphatic aldehydes and acids. Since peroxides are known to have antiseptic properties, and since recent studies by Keeney and co-workers (2) have shown the fungicidal and fungistatic action of various fatty acids, such as propionic, caprylic, and undecylenic acids, it seemed of interest to investigate the action of ozonized olive oil on bacteria and fungi. Some experiments about the bactericidal properties of ozonized olive oil have been reported by Stevens (3).

The following organisms were included in the present study: *Staphylococcus aureus, Staphylococcus albus, Streptococcus hemolyticus, Monilia albicans, Trichophyton mentagrophytes, Trichophyton purpureum, Microsporon audouini,* and *Microsporon lanosum.* The procedures employed were the agar-plate-cup and the agar-plate method using nutrient agar, blood agar, blood agar base, or Sabouraud's agar. The results were evaluated by measuring the width of the clear agar zones from the cup to outer margin of the zone.

EXPERIMENTAL

The action of ozonized olive oil on the investigated bacteria is definitely bactericidal (Table I). Control experiments with both fresh olive oil[1] and triolein showed neither bacteriostatic nor bactericidal action.

As can be expected, the bactericidal action will be influenced to a certain extent by the surrounding medium. If there are substances present which will react easily with active oxygen, the bactericidal action of the oil will be reduced. This can be visibly demonstrated in blood agar plates where a colorless zone of about 1 mm. is always formed on contact with ozonized olive oil.

The relationship between the amount of available active oxygen and the bactericidal effect is shown in Table II. From these figures it can be seen that a partly ozonized olive oil is the most suitable product for topical use. It combines the greatest bacteri-

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[1] The fact that olive oil occasionally shows a weak bacteriostatic and sometimes even bactericidal effect is due to the presence of oleic acid peroxides which are formed on prolonged exposure to air. These peroxides can be detected and estimated in the same manner as ozonides.

TABLE I.—AGAR-CUP-PLATE TESTS USING 4 DROPS OF OZONIZED OLIVE OIL CONTAINING 1% BY WEIGHT OF ACTIVE OXYGEN

Organism	Zone in Mm. after 24 Hr. Using Nutrient Agar	Blood Agar Base	Blood Agar	Subplating Tests Total No.	Showing Growth	No growth
Staph. aureus	8–9	6–7	2	18	1	17
Staph. albus	6–7	...	...	10	0	10
Strept. hemol.	10–12	5–8	2–3	27	6	21

cidal power with the deepest penetration. Higher concentrations of triolein ozonide are of no advantage because the oil becomes too viscous while lower concentrations are less effective.

The experiments in the bottom line of Table II were made with a sample of ozonized olive oil which was about two years old and had lost more than half of its original active oxygen content. The amount of decomposition products had correspondingly increased. The figures show that the bactericidal action of the sample is weaker than that of freshly made oil with the same active oxygen content. This indicates that decomposition products do not contribute noticeably to the bactericidal action of ozonized olive oil.

It is obvious that ozonized olive oil does not act instantaneously upon contact like the protein precipitating antiseptics but requires some time for the decomposition of the ozonide and liberation of active oxygen before affecting the organism. In order to test this time factor, small pieces were cut out from the innermost and outermost part of the clear agar zones and were subplated separately. The results shown in Table III are conclusive. The inner zone where the oil penetrated first and had the longest time to act upon the bacteria, was completely sterile, while in the outer part of the clear agar zone the action was only bacteriostatic. The same table also shows that the number of organisms present is an additional factor affecting the action of ozonized olive oil, as evidenced by the varying widths of the zones.

TABLE II.—AGAR-CUP-PLATE TESTS USING 2 AND 4 DROPS, RESPECTIVELY, OF OZONIZED OLIVE OIL CONTAINING VARIOUS AMOUNTS OF ACTIVE OXYGEN

Material	Active Oxygen, %	Zone in Mm. after 24 Hr. *Staph. aureus* 2 Drops of Oil	*Staph. aureus* 4 Drops of Oil	*Staph. albus* 2 Drops of Oil	*Staph. albus* 4 Drops of Oil
Freshly	2	...	13–16	...	10–12
prepared	1	10–11	13–16	10–12	16–17
ozonized	0.5	6–8	8–10	2–4	6–8
olive	0.25	2	4–5	1	2–3
oil	0.125	1	1–2	0	1
2-year-old sample	0.4	2	3–4	1–2	3–4

TABLE III.—AGAR-PLATE-CUP TESTS USING 4 DROPS OF OZONIZED OLIVE OIL CONTAINING 1% BY WEIGHT OF ACTIVE OXYGEN

24-Hr. Broth Culture of *Staph. aureus*	Zone in Mm. after 24 Hr.	Growth in Subplating Tests Samples Taken From Innermost Part of Clear Agar Zone[a]	Outermost Part of Clear Agar Zone[a]
Undiluted	6–7	–, –, –	+, +, +
Diluted 1:10	8–9	–, –, –	–, +, +
Diluted 1:100	11–12	–, –, –	+, +, +

[a] Each + or – sign represents the result of a separate subplating test.

In considering an antiseptic from a practical clinical standpoint, it is important to know whether the agent is able to kill bacteria after they had a chance to grow and multiply. In order to test for this condition, agar plates containing bacteria were incubated for a given time before the ozonized olive oil was added. The results (Table IV) show a very definite though decreasing effect up to a preincubation time of six hours.

TABLE IV.—AGAR-PLATE-CUP TESTS IN WHICH 4 DROPS OF OZONIZED OLIVE OIL CONTAINING 1% BY WEIGHT OF ACTIVE OXYGEN WERE ADDED AFTER VARYING PREINCUBATION TIMES

Preincubation time, Hr.	Zone in Mm. 24 Hr. after Adding Ozonized Olive Oil *Staph. aureus*	*Staph. albus*	*Strep. hemol.*
1	4	4	3
2	4	3	...
3	4	2–3	2–3
4	3–4	2	1
6	1–2	0	0
8	0	0	0

The fungi which were tested in this investigation are *Monilia albicans, Trichophyton mentagrophytes, Trichophyton purpureum, Microsporon audouini* and *Microsporon lanosum*. The first three were selected because they are some of the most common organisms in epidermophytosis (4), while the latter two are the causative agents in ringworm of the scalp. The results are summarized in Table V.

Under the tested conditions, ozonized olive oil has a strong fungicidal action against *Trichophyton purpureum, Trichophyton mentagrophytes, Microsporon audouini*, and *Microsporon lanosum*. Against *Monilia albicans* it has only fungistatic action.

Since the growth rate of the fungi used in these experiments is comparatively low, the time effect might be of considerable importance. Therefore, a series of experiments was made like those with bacteria. Agar plates containing the fungi were incubated until an abundant growth was present. Then the plates were covered completely with ozonized olive oil, and incubated for a period ranging from twenty-four to seventy-two hours. After this time, samples were taken out, freed from the oil, and transferred to new sterile plates. The results

TABLE V.—AGAR-PLATE-CUP TESTS USING 4 DROPS OF OZONIZED OLIVE OIL CONTAINING 1% BY WEIGHT OF ACTIVE OXYGEN

Organism	Incubation Time, Hr	Temp., °C.	Zone, Mm	Subplating Tests[a]
Monilia albicans	48	20	4–5	+ + + + + +
	48	20	3–4	+ + + + + +
	48	20	3–4	+ + + + + +
Trichophyton mentagrophytes	168	20	16–18	– – – – – –
	96	25	18–23	– – – – – –
	72	25	20–24	– – – – + +
	72	25	16–20	– – – – + +
Trichophyton purpureum	240	20	19–24	– – – – – –
	168	25	18–23	– – – – – –
	120	25	12–17	– – – – – –
	72	25	14–16	– – – – – –
Microsporon audouini	96	35	18–23[b]	– – – – – –
	96	35	22–26	– – – – – –
Microsporon lanosum	96	35	8–11[b]	– – – – – –
	96	35	11–15	– – – – – –

[a] Each + or – sign represents the results of a separate subplating test. Samples for the upper 3 tests of each series were taken from the innermost and those for the lower 3 tests from the outermost part of the clear zone.
[b] Only 2 drops of ozonized olive oil used.

as shown in Table VI are again very uniform. The ozonized olive oil will, under these conditions, kill completely all the investigated fungi with exception of *Monilia albicans*. Control samples in which olive oil was used instead of the ozonized oil showed in all cases abundant growth of the organism.

DISCUSSION

From the foregoing experiments, it is apparent that ozonized olive oil has considerable germicidal potency. Although it shares this property with a great number of other chemical compounds, it is quite unique in various respects.

Triolein ozonide itself is lipoid soluble while its decomposition products are water soluble. In addition it has a low surface tension and does not dry out thus preventing any caking or crusting if applied topically. Therefore, one can expect that ozonized olive oil is able to penetrate to places which are inaccessible to many other antiseptics. It is apparently this combination of physical and chemical properties which accounts to a large extent for the numerous beneficial clinical results some of which have already been reported [Greenberger and Helfert (5), Barrows (6), Sharlit (7)].

It has been mentioned that the bactericidal action of ozonized olive oil is due mainly to the active (nascent) oxygen which is formed on decomposition of the ozonides. Undoubtedly, this active oxygen plays also an important role in the fungicidal activity of this material. In addition, however, one must also consider two of the decomposition products of ozonized olive oil, *viz.*, pelargonic and caproic acid. The latter has been shown by Keeney, *et al.* (2), to have fungistatic and fungicidal properties against a variety of organisms. More important because it is pres-

TABLE VI.—AGAR PLATES COVERED WITH OZONIZED OLIVE OIL AND OLIVE OIL, RESPECTIVELY, AFTER VARYING PREINCUBATION TIMES

Organism	Incubation Time in Hours: Before Adding Test Substance	After Adding Test Substance	Growth in Subplating Tests[a] When the Substance Was: Ozonized Olive Oil	Olive Oil
Monilia albicans	24	24	+ + +	+ + +
	24	48	+ + +	+ + +
	24	72	+ + +	+ + +
Trichophyton mentagrophytes	48	24	– – –	+ + +
	48	72	– – –	+ + +
Trichophyton purpureum	72	24	– – –	+ + +
	72	48	– – –	+ + +
	72	72	– – –	+ + +
Microsporon audouini	96	24	– – –	+ + +
	96	48	– – –	+ + +
	96	72	– – –	+ + +
Microsporon lanosum	96	24	– – –	+ + +
	96	48	– – –	+ + +
	96	72	– – –	+ + +

[a] Each + or – sign represents the results of a separate subplating test.

ent in greater quantities is pelargonic acid. In very recent investigations Rothman and co-workers (8) have found that this acid is a normal constituent in hair fat of adults while it is apparently lacking in children. Furthermore, pelargonic acid has a strong fungicidal activity against *Microsporon audouini*. Based on these findings the authors postulate that the absence of the ringworm of the scalp in adults is due to the appearance of pelargonic acid in hair fat during adolescence.

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Microscopic Description of the Antihistamine Substances Benadryl and Pyribenzamine*

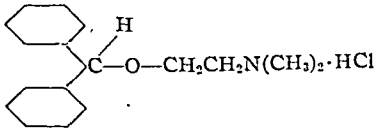
By GEORGE L. KEENAN†

The optical crystallographic properties of benadryl and pyribenzamine have been described, together with significant microchemical reactions with platinic chloride reagent.

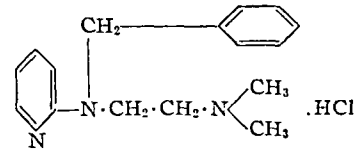
BENADRYL and pyribenzamine have both aroused considerable interest in clinical medicine as antihistamine substances. It is known that histamine is normally present in most of the organs which display allergic manifestations and its presence accounts for many of the symptoms of allergic reactions. Considerable work has been done and reported (1–4) on the effects of a series of compounds with respect to their interference with the action of histamine.

The purpose of this report is to place on record the results of a microscopic study of these two compounds, applying the technic of the immersion method as well as microchemical tests.

Benadryl[1] is β-dimethylaminoethyl benzhydryl ether hydrochloride:



Pyribenzamine hydrochloride[2] is N′-pyridyl-N′-benzyl-N-dimethylethylene diamine hydrochloride:



Both of these compounds are suitable for optical and microchemical study, showing many distinctive features that are useful for

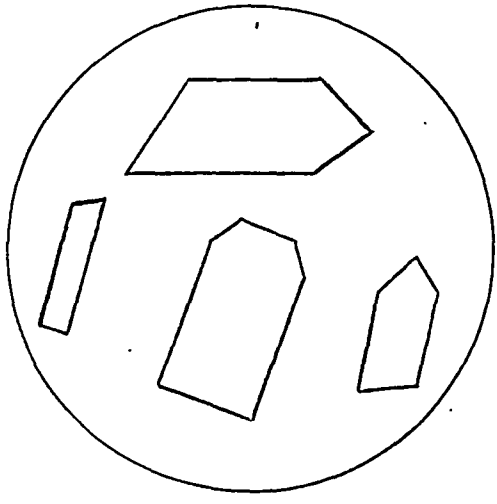


Fig. 1—Benadryl (typical habits).

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† Formerly Microanalyst, Food & Drug Administration; present address, Strongsville, Ohio.
[1] Furnished through the courtesy of Parke, Davis & Company, Detroit, Mich.
[2] Furnished through courtesy of Ciba Pharmaceutical Products, Inc., Summit, N. J.

diagnosis. For the optical crystallographic study, the immersion method with the polarizing microscope was used. The microchemical tests were made by dissolving the respective compounds in a suitable menstruum, then drawing in a drop of the appropriate reagent. For the significant microscopic characters, the optical crystallographic constants first will be given, followed by a description of the microchemical tests.

EXPERIMENTAL

Benadryl.—Benadryl consists of a white crystalline substance, quite soluble in water and alcohol and stable under ordinary conditions.

In ordinary light: Colorless, most frequently in six-sided plates with lengthwise cleavage (Fig. 1).

Refractive indices: $\alpha = 1.602$, $\beta = 1.625$, $\gamma = 1.630$, all $\pm$ 0.002. Most plates show α lengthwise and β and γ crosswise (Fig. 2).

In parallel polarized light (crossed nicols): Extinction is parallel and the sign of elongation is negative.

In convergent polarized light (crossed nicols): Biaxial but only flash interference figures are shown. Optic sign (−)

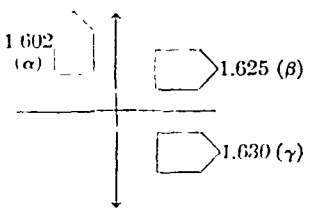

Fig. 2—Benadryl (orientation).

Microchemical Test.—A significant microchemical test for benadryl is platinic chloride reagent (5 Gm. of $H_2PtCl_6 \cdot 6H_2O$ in 100 ml. of H_2O). The most convenient method for making the test is by dissolving approximately 0.5 mg. of the material in a drop of water on an object slide, then drawing in a drop of the platinic chloride reagent. Gradually X-shaped aggregates of very thin blades or leaf-like structures are produced. These blades show a line passing through the center, the general appearance of the structure simulating that of a leaf with midrib. Due to the thinness of the blades, the polarization colors are not at all brilliant (Fig. 3).

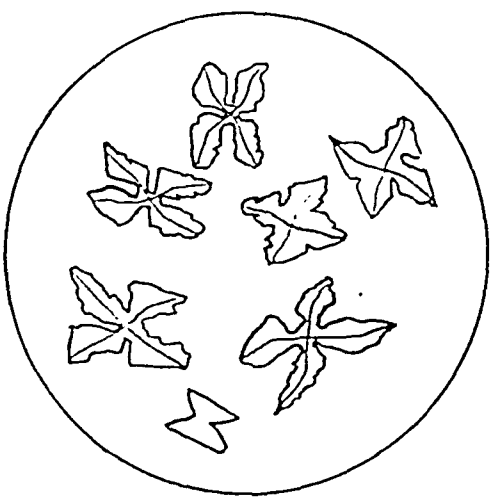

Fig. 3—Benadryl with Platinic chloride.

Pyribenzamine.—Pyribenzamine hydrochloride consists of a white, crystalline powder, the habit of the fragments not being significant. It is very soluble in water and when crystallized from a concentrated drop on an object slide shows many rectangular plates and prisms exhibiting striking interference colors with crossed nicols.

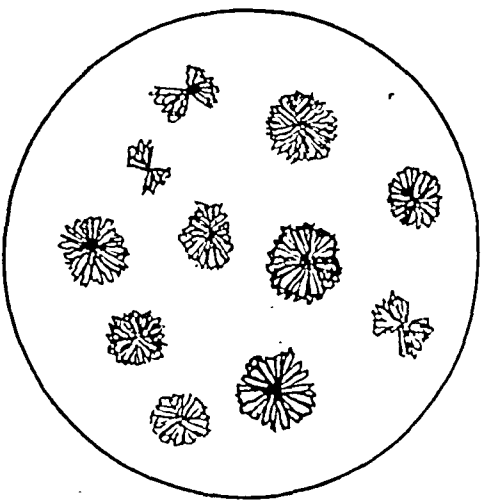

Fig. 4—Pyribenzamine with Platinic chloride.

Refractive indices: $\alpha = 1.580$, $\beta = 1.655$, $\gamma = 1.705$, all $\pm$ 0.002. All of the indices are readily found on the substance.

In parallel polarized light (crossed nicols): It is characteristic of the substance that many fragments do not extinguish sharply.

In convergent polarized light (crossed nicols): Biaxial, optic axis figures being commonly shown. Optic sign (−).

Microchemical Test.—With platinic chloride reagent, pyribenzamine forms dense rosettes and sheaves of small rods or blades. The reagent is drawn into the test-drop (about 0.5 mg. in a drop of water) on an object slide, whereupon the characteristic aggregates form (Fig. 4).

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Studies on Alcohol-Soluble Fungistatic and Fungicidal Compounds. I. Evaluation of Fungistatic Laboratory Test Methods*,†

By KURT A. OSTER and MILTON J. GOLDEN

The two principal methods for the determination of fungistasis have been compared and their limitations noted. From the dosage response curves gained by this study a new expression is suggested, the "activity coefficient," to denote degree of fungistatic activity exhibited by a substance. Ten compounds extensively employed in the therapy of dermatophytosis have been tested with *Trichophyton mentagrophytes*, and their relative potencies have been determined.

BURLINGAME and Reddish in 1939 (1) brought the subject of laboratory testing of fungistatic and fungicidal substances up to that date and suggested a new test method, following in principle the serum agar-cup-plate technique described in the U. S. Department of Agriculture Circular No. 198 (2). However, aside from the statement that their findings ran closely parallel with clinical observations, this paper made no mention of actual controls or of any limitations in their method. Schamberg and Kolmer (3) had suggested in 1922 a fungistatic test method to which Burlingame and Reddish objected, claiming that it did not simulate clinical conditions.

In 1945 the Council on Pharmacy and Chemistry of the American Medical Association issued a report (4) in which it was declared urgent that a standard test for fungistatic and fungicidal substances be established. Use of the fungicidal test known as the phenol coefficient test for disinfectants and antiseptics, as modified by the American Public Health Association subcommittee, was suggested. However, it was noted that the test is meaningful only for surface growth, such as "testing the action of fungicides on floors and the like." Furthermore, it was stated that the problem of testing nonwater-soluble products presents even greater difficulties.

In view of the unsettled status of these test procedures, it was considered a desirable undertaking to subject to critical laboratory evaluation the principally used alcohol-soluble compounds for the therapy of foot dermatophytosis and also the existing test methods by which the comparative fungistatic potencies of these compounds are assayed.

METHODS

Following are the outlines of the methods used:

Burlingame and Reddish (1).—Solutions are tested by the agar-cup-plate technique. The serum agar is prepared by adding 2 cc. of sterile normal horse serum to 18 cc. of Sabouraud's agar, which has been melted and cooled to 40°. The agar is poured into a 9-cm. Petri dish, and, when hardened, the entire surface of each plate is streaked with a ten-day-old culture of the test organism. A 2-cm. cup is cut from the center of the plate with a sterile cork borer, and approximately 0.8 cc. of the test fluid is pipetted into the cup. The plates are then covered with unglazed clay tops to prevent condensation and incubated for five days. The fungistatic activity of a given compound is indicated by a cleared zone around the cup. Solvent controls with 95% alcohol accompanied each series of determinations. The fungistatic effect of the alcohol in this test method was found to be minimal.

Modified Burlingame and Reddish (5).—A modification of the agar-cup-plate technique has been suggested by substituting incorporation of a ten-day-old conidial suspension in the agar for the procedure of streaking a fungus culture on the surface of the agar plate. A 1-cc. suspension of approximately five million spores from a ten-day-old culture, counted in a haemocytometer and diluted with saline, is thoroughly mixed with 15 cc. of Sabouraud's agar, which had been melted and cooled to 40°. An alcoholic solution of the test substance is pipetted into a 2-cm. cup, cut and removed from the center of the hardened agar. The plates, covered with porous clay tops, are incubated for 96 hours, at which time the clear zone around the cup, indicating inhibition of growth, is measured.

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Presented in part before the Division of Biological Chemistry of the American Chemical Society at its semiannual meeting, Atlantic City, N.J., April, 1946.

† A recent publication by Hillegas and Camp (10) on the testing of fungicides insoluble in water came to our attention after the initiation of this work. These authors express ideas on the comparative measurement of fungistatic compounds which are similar to those propounded in our present study. However, their suggested "penicup" method for determining fungistasis was not reproducible in our hands.

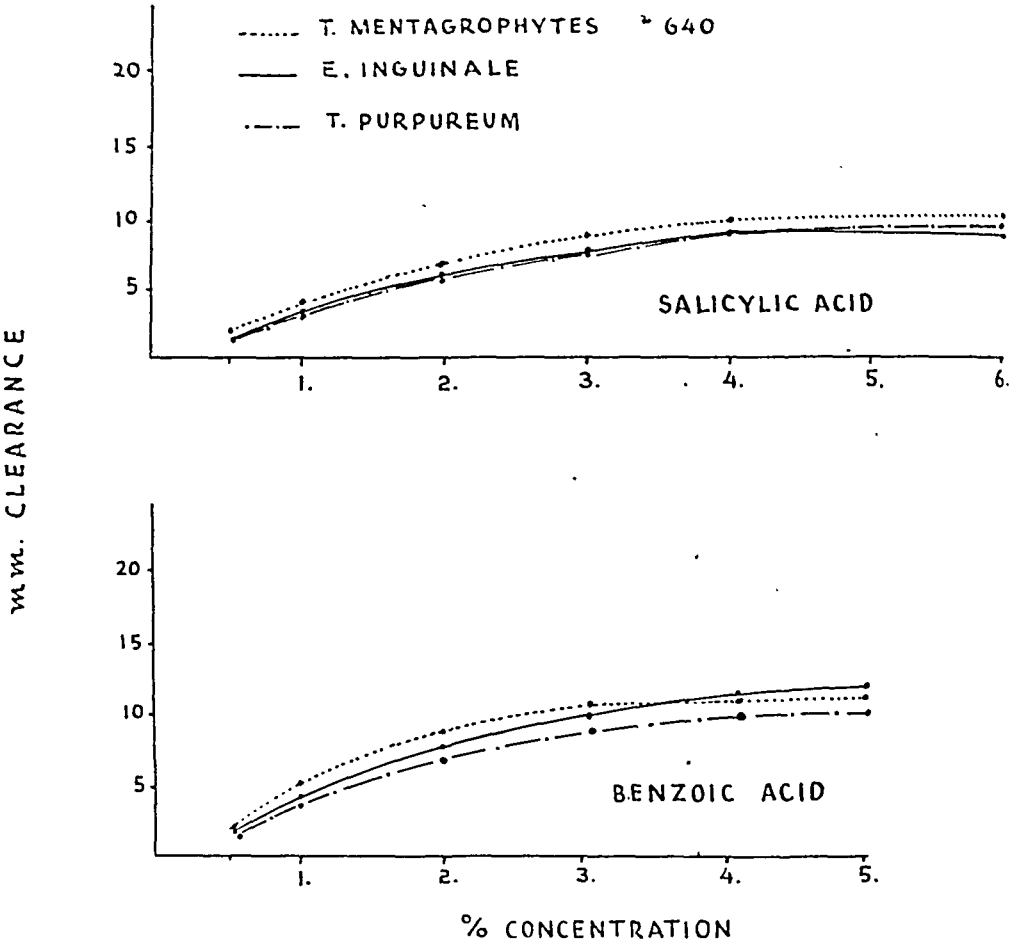


Fig. 1.—Dosage and fungistatic response curves of three species of fungi with increasing concentrations of salicylic acid and benzoic acid.

Schamberg and Kolmer (3).—This method for determining the fungistatic effect of water-soluble substances has been so modified as to be applicable to alcohol-soluble compounds without violating its underlying principles. In view of the fact that alcohol itself possesses minor fungistatic properties, it was necessary to limit the quantity of alcohol incorporated in the agar to 0.1 cc., an amount which exerted no noticeable fungistasis. Plates were poured, each containing 20 cc. of Sabouraud's agar into which 0.1 cc. of an alcoholic solution of the test substance had been incorporated, thereby diluting by two hundred times the original concentration of the test substance in alcohol. The surfaces of the plates were streaked with a ten-day-old culture of the test organism, and the plates were incubated for two weeks, after which time the presence or absence of growth was noted. The highest dilution of the test material which completely inhibited the growth of fungi after a two-week incubation period was regarded as the critical fungistatic dilution.

The 3 fungi commonly associated with dermatophytosis of the human foot, *Epidermophyton inguinale*, *Trichophyton purpureum* and *Trichophyton mentagrophytes* (*T. interdigitale*, *T. gypseum*), were first tested individually. *T. mentagrophytes* (#640 Emmons) was later chosen as the sole test organism

The incubator temperature for all tests was 28° ±1°. Stock cultures of the fungus were stored on maltose agar slants at 2–5°. At intervals not longer than one month transfers were made to fresh agar slants, incubated at 28° for at least ten days, and placed in storage at 2–5° until the next transfer period. It was ascertained with the phenol coefficient technique (6) that a spore suspension of a ten-day-old culture of *T. mentagrophytes* was killed after contact with phenol for ten minutes at a concentration of 1:60 and not by a concentration of 1:80.

The following 10 active compounds commonly used in the therapy of dermatophytosis were chosen for study: benzoic acid, salicylic acid, phenol, resorcinol, cresol U. S. P., metacresylacetate, thymol, chlorothymol, propionic acid, and undecylenic acid.[1] Of the 103 marketed athlete's foot remedies with declared formulas, as collected by Underwood,

[1] Undecylenic acid supplied by Dr. L. Reiner, Wallace & Tiernan Products, Inc., Belleville, N. J.

et al. (7), 76 contained as active ingredients from 1 to 4 of these compounds under test.

RESULTS

Using 5 determinations for each organism and for each concentration with the Burlingame and Reddish method, reproducible inhibition zones for the evaluation of the fungistatic action of the above-mentioned compounds have been obtained in a range of critical concentration. The inhibition of growth around the cup was measured in millimeters from the edge of the cup to the periphery of the cleared zone, and averages of the 5 measurements of these radius segments were calculated.

Approximately 400 individual determinations were made with various concentrations of the 10 compounds. It was established that the fungistatic values obtained with the examples of salicylic acid and benzoic acid for the 3 test organisms, *T. mentagrophytes*, *E. inguinale*, and *T. purpureum*, ran closely parallel. In subsequent tests *T. mentagrophytes* (#640 Emmons) was chosen as the representative test organism to save time and to reduce the number of tests. It was assumed that *E. inguinale* and *T. purpureum* would give with the remaining 8 compounds values approximating fairly closely those of *T. mentagrophytes*.

It was observed that the cleared zones exhibited the greatest variations with either very low or very high concentrations of the test substance. Values falling between the 2 extremes never deviated more than ±1 mm. Typical dosage response curves were obtained by plotting the radius segments of clearance against the respective concentrations. These findings are shown in Figs. 1 and 2. The shape of each curve was determined by at least 5 points at various concentrations. In some instances only a

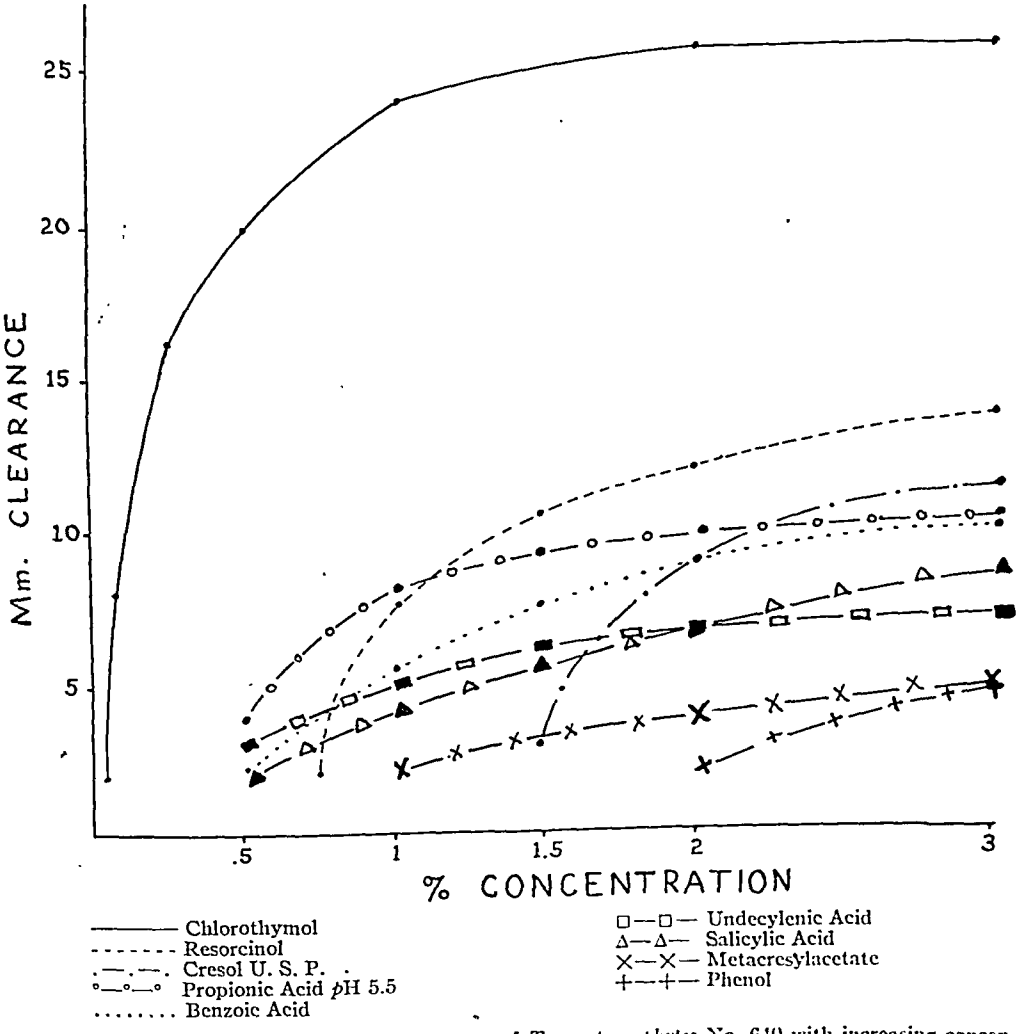
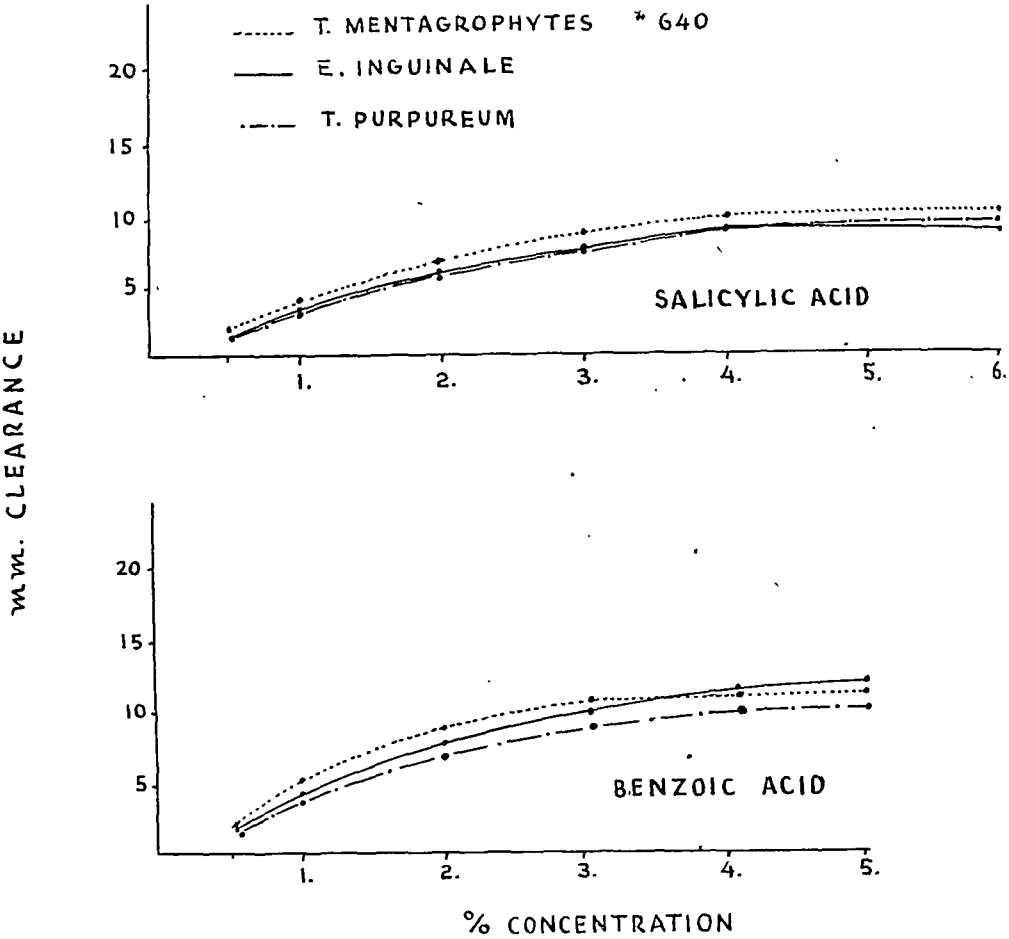


Fig. 2.—Dosage and fungistatic response curves of *T. mentagrophytes* No. 640 with increasing concentrations of various alcohol soluble compounds.



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Approximately 400 individual determinations were made with various concentrations of the 10 compounds. It was established that the fungistatic values obtained with the examples of salicylic acid and benzoic acid for the 3 test organisms, *T. mentagrophytes*, *E. inguinale*, and *T. purpureum*, ran closely parallel. In subsequent tests *T. mentagrophytes* (#640 Emmons) was chosen as the representative test organism to save time and to reduce the number of tests. It was assumed that *E. inguinale* and *T. purpureum* would give with the remaining 8 compounds values approximating fairly closely those of *T. mentagrophytes*.

It was observed that the cleared zones exhibited the greatest variations with either very low or very high concentrations of the test substance. Values falling between the 2 extremes never deviated more than ±1 mm. Typical dosage response curves were obtained by plotting the radius segments of clearance against the respective concentrations. These findings are shown in Figs. 1 and 2. The shape of each curve was determined by at least 5 points at various concentrations. In some instances only a

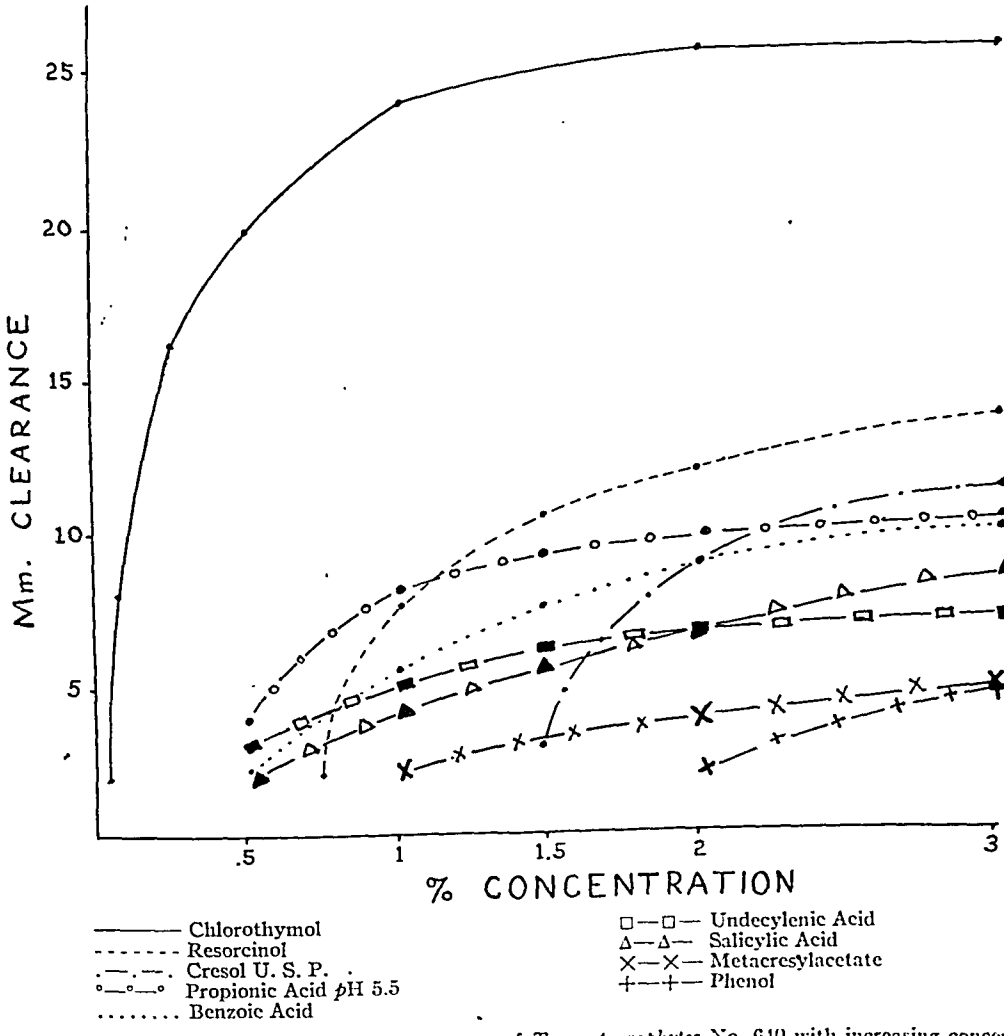

Fig. 2.—Dosage and fungistatic response curves of *T. mentagrophytes* No. 640 with increasing concentrations of various alcohol soluble compounds.

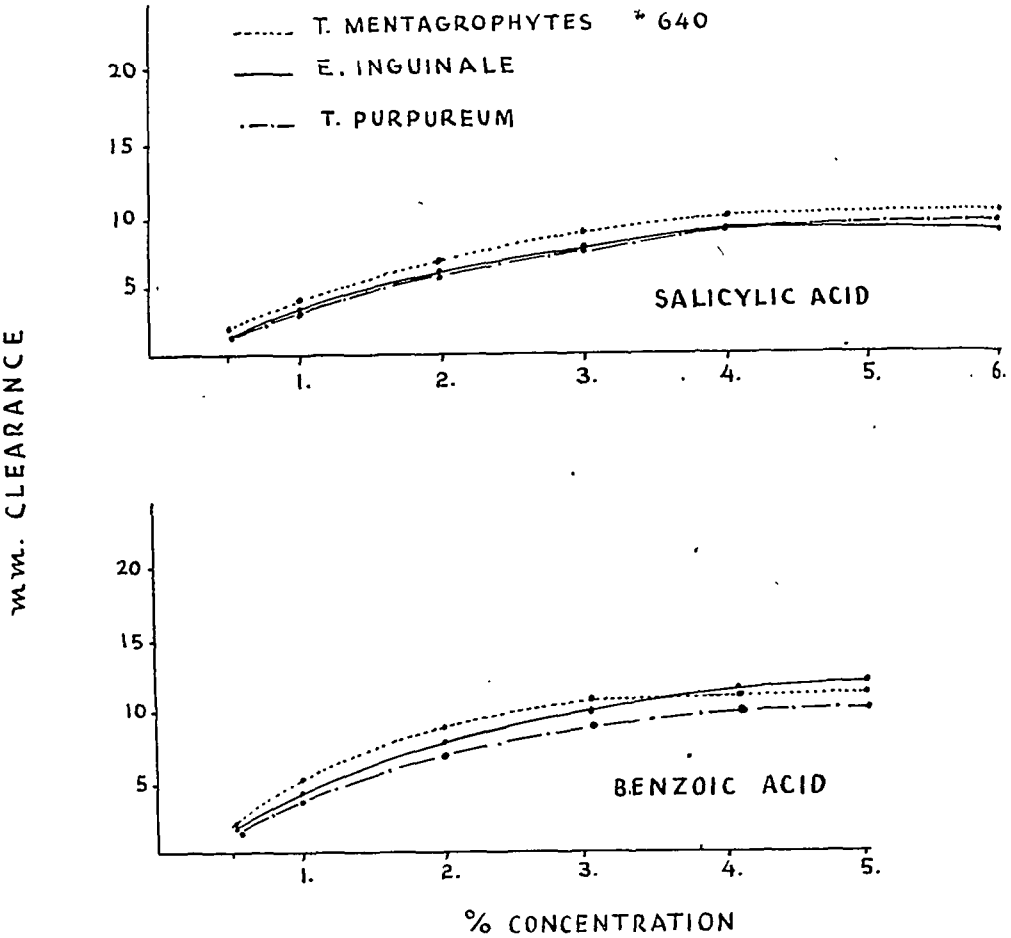

Fig. 1.—Dosage and fungistatic response curves of three species of fungi with increasing concentrations of salicylic acid and benzoic acid.

Schamberg and Kolmer (3).—This method for determining the fungistatic effect of water-soluble substances has been so modified as to be applicable to alcohol-soluble compounds without violating its underlying principles. In view of the fact that alcohol itself possesses minor fungistatic properties, it was necessary to limit the quantity of alcohol incorporated in the agar to 0.1 cc., an amount which exerted no noticeable fungistasis. Plates were poured, each containing 20 cc. of Sabouraud's agar into which 0.1 cc. of an alcoholic solution of the test substance had been incorporated, thereby diluting by two hundred times the original concentration of the test substance in alcohol. The surfaces of the plates were streaked with a ten-day-old culture of the test organism, and the plates were incubated for two weeks, after which time the presence or absence of growth was noted. The highest dilution of the test material which completely inhibited the growth of fungi after a two-week incubation period was regarded as the critical fungistatic dilution.

The 3 fungi commonly associated with dermatophytosis of the human foot, *Epidermophyton inguinale*, *Trichophyton purpureum* and *Trichophyton mentagrophytes* (*T. interdigitale*, *T. gypseum*), were first tested individually. *T. mentagrophytes* (#640 Emmons) was later chosen as the sole test organism.

The incubator temperature for all tests was 28° ±1°. Stock cultures of the fungus were stored on maltose agar slants at 2–5°. At intervals not longer than one month transfers were made to fresh agar slants, incubated at 28° for at least ten days, and placed in storage at 2–5° until the next transfer period. It was ascertained with the phenol coefficient technique (6) that a spore suspension of a ten-day-old culture of *T. mentagrophytes* was killed after contact with phenol for ten minutes at a concentration of 1:60 and not by a concentration of 1:80.

The following 10 active compounds commonly used in the therapy of dermatophytosis were chosen for study: benzoic acid, salicylic acid, phenol, resorcinol, cresol U. S. P., metacresylacetate, thymol, chlorothymol, propionic acid, and undecylenic acid.[1] Of the 103 marketed athlete's foot remedies with declared formulas, as collected by Underwood,

[1] Undecylenic acid supplied by Dr. L. Reiner, Wallace & Tiernan Products, Inc., Belleville, N. J.

et al. (7), 76 contained as active ingredients from 1 to 4 of these compounds under test.

RESULTS

Using 5 determinations for each organism and for each concentration with the Burlingame and Reddish method, reproducible inhibition zones for the evaluation of the fungistatic action of the above-mentioned compounds have been obtained in a range of critical concentration. The inhibition of growth around the cup was measured in millimeters from the edge of the cup to the periphery of the cleared zone, and averages of the 5 measurements of these radius segments were calculated.

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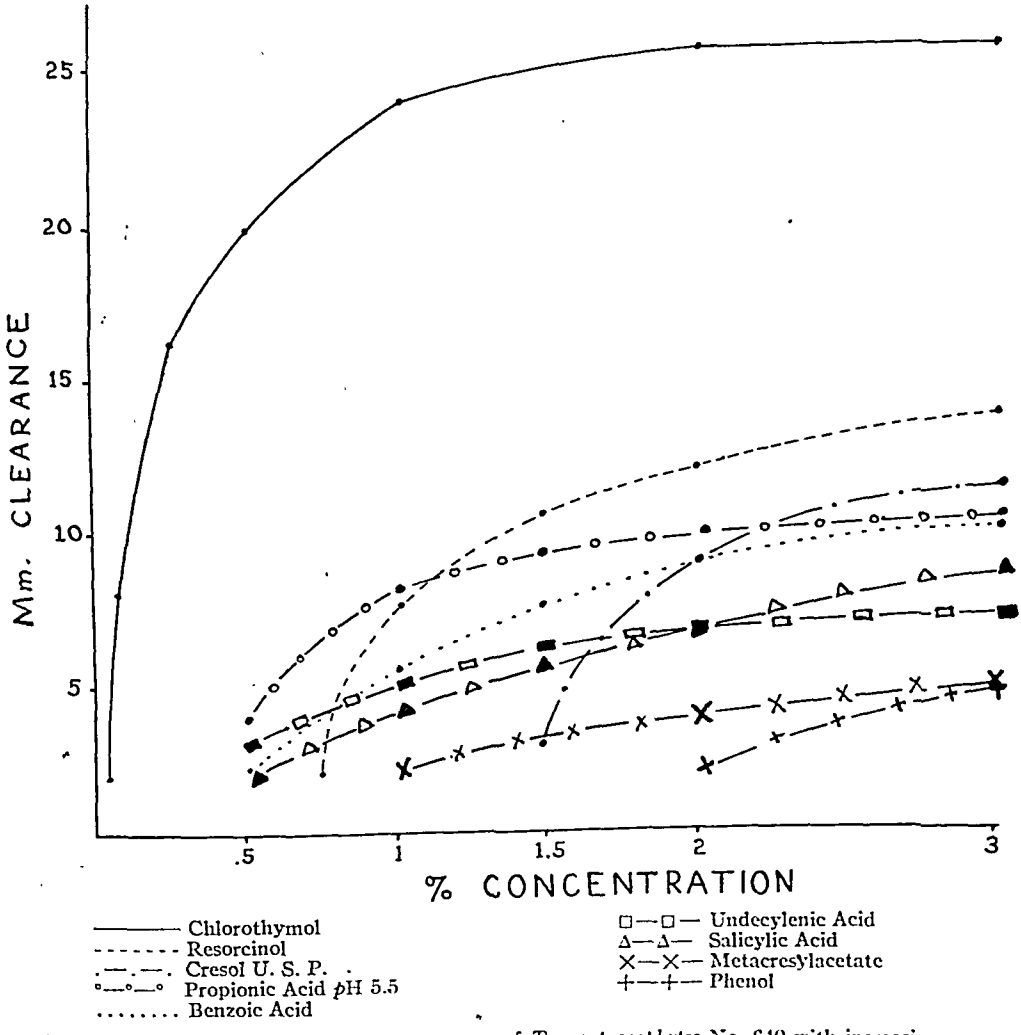

Fig. 2.—Dosage and fungistatic response curves of *T. mentagrophytes* No. 640 with increasing concentrations of various alcohol soluble compounds.

portion of the curves is presented, because values in excess of 3% concentration were eliminated in the illustration. From these dosage response curves it appeared that all the substances tested exhibited a ceiling of activity. Once this ceiling had been reached, an increase in concentration did not induce a corresponding rise in fungistasis.

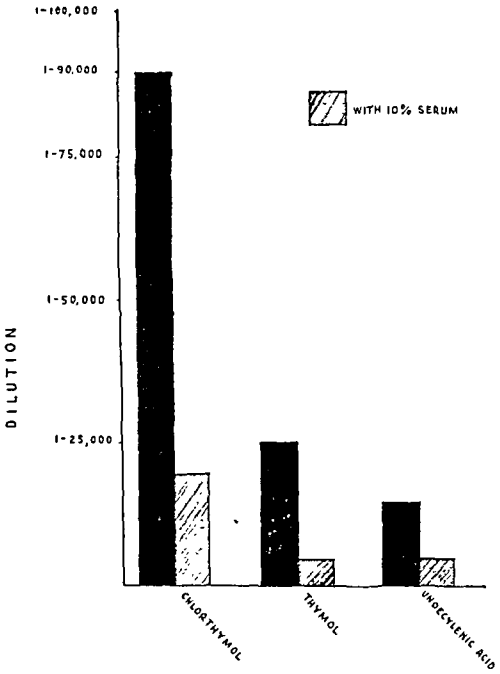

Fig. 3.—Maximum dilution of alcohol soluble fungistatic compounds inhibiting the growth of *T. mentagrophytes* No. 640 on Sabouraud's maltose agar.

Following the line of reasoning behind the establishment of the Oxford unit for expressing penicillin activity (8), the introduction of dosage response curves in the determination of fungistasis might facilitate the evaluation of the fungistatic activities of compounds by expressing their respective activity coefficients. A standard of 5-mm. clearance at a 1% concentration was arbitrarily chosen as the activity 1. Using *T. mentagrophytes* as the test organism, the concentration at which a compound exhibited 5-mm. clearance divided into the figure 1 gave then the fungistatic activity coefficient of this particular substance. The values thus obtained were approximated to the nearest tenth and listed in Table I. Substituting molar concentration for per cent concentration, the position of propionic acid and undecylenic acid would be reversed in the table. The order of the other substances would remain the same. Substances which do not reach 5-mm. clearance even in higher concentrations could not be evaluated by this method. They would also most likely fall short of any therapeutic usefulness.

Using the described modification of the Burlingame and Reddish technique (5), which requires incorporation of a conidial suspension with the agar, gave much the same results with the compounds as encountered with the original Burlingame and Reddish procedure of culture streaking. The zone of inhibiton of growth was slightly larger than that observed with the streaking method. Furthermore, the cleared zone was almost a perfect circle, a result which was not always obtainable with the Burlingame and Reddish technique. However, these advantages were outbalanced by the inherent cumbersomeness of the method, which requires standardization, counting, and continuous availability of a suitably aged spore suspension. This is extremely time consuming, especially in large-scale routine testing of substances. Sealing the cup was found to be unnecessary, provided the agar medium was not cracked, since equal inhibition was obtained with both sealed and unsealed plates. The greater clearance shown by this modification might be due to the absence of serum in the agar medium, which greatly inhibited the fungistatic action of certain chemicals.

The more volatile compounds in our series, thymol, cresol, and metacresylacetate, were difficult to test with the Burlingame and Reddish technique. Thymol was finally eliminated from the series due to unreproducible results. Undiluted metacresylacetate caused a whitish discoloration of the agar, and no growth was demonstrable, even when using sterile filter paper supports for the porous plates to enhance evaporation. Cresol U. S. P. and metacresylacetate in various dilutions up to 3% inhibited to a great extent the surface growth of the Trichophytons, so that with these compounds only subsurface development could be measured. Moreover, when using concentrations greater than 3%, it was impossible to obtain reproducible values with these compounds.

TABLE I.—ACTIVITY COEFFICIENTS OF ALCOHOL-SOLUBLE FUNGISTATIC COMPOUNDS

Compound	% Conc. of Test Solution to Give 5-Mm. Clearance	Activity Coefficient
Chlorothymol	0.0625	16.0
Propionic acid	0.60	1.7
Resorcinol	0.81	1.2
Benzoic acid	0.91	1.1
Undecylenic acid	1.0	1.0
Salicylic acid	1.28	0.8
Cresol U. S. P.	1.58	0.6
Metacresylacetate	3.35	0.3
Phenol	4.0	0.25

Another method generally employed with minor modifications to demonstrate fungistasis is that originally proposed by Schamberg and Kolmer (3), where the test substance is incorporated with the agar medium which is then streaked with the test organism. Findings with this method, comparing the fungistatic activities of the previously mentioned 10 compounds, are charted in Figs. 3 and 4.

This test reveals the outstanding action of chlorothymol, which is fungistatic for *T. mentagrophytes* in dilutions of up to 1:90,000. This substance is followed by thymol, 1:25,000, and undecylenic acid, 1:15,000. The remainder of the compounds displayed no remarkable activity, their fungistatic activities appearing in dilutions of from 1:2500 to 1:1500.

The medium suggested by Schamberg and Kolmer contained no serum. When normal horse serum was added to the medium to give a concentration of 10%, the fungistatic action of 7 of the test compounds was remarkably curtailed. Chlorthymol lost 78% of its fungistatic power, dropping from 1:90,000 to 1:20,000. Only cresol, metacresylacetate, and phenol were not affected by the addition of serum.

Peck and Rosenfeld (9) claimed ascorbic acid to be fungistatic. This statement could not be confirmed by us. It was found that ascorbic acid dissolved in 95% alcohol exhibited no zone of growth inhibition even at a concentration of 2% with the Burlingame and Reddish method. Using the Schamberg and Kolmer method, it did not prevent the growth of *T. mentagrophytes* in dilutions of up to 1:500.

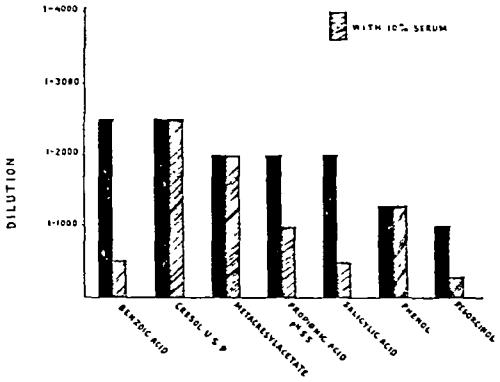

Fig. 4.—Maximum dilution of alcohol soluble fungistatic compounds inhibiting the growth of *T. mentagrophytes* No. 640 on Sabouraud's maltose agar.

DISCUSSION

The need for the establishment of a standard test for fungistatic substances has long been felt. The present study has attempted to evaluate the fungistatic potencies of ten compounds commonly used in the therapy of dermatophytosis. The results observed by utilizing the two principal test methods as suggested by Burlingame and Reddish and by Schamberg and Kolmer were compared. On arranging the ten test compounds in an order of decreasing fungistatic activity, no correlation could be noted between the results obtained with the two methods. See Table II.

The Schamberg and Kolmer procedure proved to be quite helpful in investigating the effects of volatile substances, which could not be accomplished with the Burlingame and Reddish technique. However, the former method demonstrated but one value for each compound; either there was growth or there was fungistasis. Nothing could be learned of eventual penetration or of ceiling of activity. It is the impression of the authors that the two techniques supplement each other and that each will be useful in its own right for evaluating the mechanism of action of fungistatic substances.

A new means of expressing fungistasis by measuring the activity coefficient derived from dosage response curves is suggested by the authors. Thus, the standardization of the activity of one substance against the activity of another, which might act by a completely different mechanism is avoided. The standard conditions would be those of medium and of culture. It appears that *T. mentagrophytes* (#640 Emmons) ade-

TABLE II.—COMPARISON OF TEN COMPOUNDS ARRANGED IN A DECREASING ORDER OF FUNGISTATIC ACTIVITY DERIVED FROM THE BURLINGAME AND REDDISH AND THE SCHAMBERG AND KOLMER METHODS

Burlingame and Reddish (Fig. 2)		Schamberg and Kolmer (Figs. 3 and 4)	
Activity Coefficient	Height of Ceiling of Dosage Response Curve	Without Serum	With Serum
Chlorothymol	Chlorothymol	Chlorothymol	Chlorothymol
Propionic acid	Resorcinol	Thymol	Thymol
Resorcinol	Cresol U. S. P.	Undecylenic acid	Undecylenic acid
Benzoic acid	Propionic acid	Benzoic acid	Cresol U. S. P.
Undecylenic acid	Benzoic acid	Cresol U. S. P.	Metacresylacetate
Salicylic acid	Salicylic acid	Metacresylacetate	Phenol
Cresol U. S. P.	Undecylenic acid	Propionic acid	Propionic acid
Metacresylacetate	Metacresylacetate	Salicylic acid	Benzoic acid
Phenol	Phenol	Phenol	Salicylic acid
		Resorcinol	Resorcinol

quately fulfills the conditions of a standard culture. For one and one-half years in this laboratory this strain has given reproducible results with all compounds under test.

Laboratory evaluation of substances designed for the therapy of dermatophytosis should be supplemented by fungicidal tests. A study of these will be published shortly.

SUMMARY

1. Results and limitations of two laboratory test methods for determining fungistasis, devised by Burlingame and Reddish and by Schamberg and Kolmer, were compared by studying the fungistatic activities of ten alcohol-soluble compounds on cultures of *T. mentagrophytes* (#640 Emmons).

2. The introduction of dosage response curves in determining fungistasis suggested a new expression of fungistatic activity, the activity coefficient.

3. The value of the two methods in the determination of fungistasis has been discussed.

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Book Review

Vitamins and Hormones, Volume IV. Edited by ROBERT S. HARRIS and KENNETH V. THIMANN, Academic Press Inc., New York, 1946. xvii + 406 pp. 14.5 x 23 cm. Price $6.80.

This is the 4th annual review of the literature in the vitamin and hormone field. When the first review was published in 1943 many no doubt wondered at the necessity for reviews published in such diversified fields. More and more, however, it is becoming evident that the two fields overlap. No doubt this will become even more evident as more research is done on the influence of the hormones on intermediary metabolism.

The first review, by Pfiffner and Hogan, is on "The Newer Hematopoietic Factors of the Vitamin B-complex." This is a very interesting review of folic acid, vitamins B_c, M, and other identical or closely related substances having folic acid activity. The authors give a number of ultraviolet absorption curves which show how closely most of these factors are interrelated. The enzyme, Vitamin B_c conjugase, which splits vitamin B_c from its conjugate is also covered. The relationship of these substances to Xanthopterine from butterflies' wings is of considerable historic interest. The authors cite 122 references.

Dr. Howard A. Schneider of Rockefeller Institute summarizes very succinctly "Nutrition and Resistance to Infection." It is here pointed out that nutritional factors may contribute either to susceptibility of, or resistance to, infection and that the action of any specific diet may well be the resultant of these opposed forces.

"Manifestations of Nutritional Deficiency in Infants," by F. W. Clements, is of interest to those working in pediatrics.

No doubt the chapter on "Nutritional Therapy of Endocrine Disturbances," by Morton S. Biskind, will cause considerable comment for its unorthodox point of view. Of equal interest will be the chapter on "The Thyroid and Diabetes," by Bernardo A. Houssay, which may be supplemented by "Thyroactive Iodinated Proteins," by E. P. Reinecke.

To pharmacists probably the most important chapter is on "Methods of Bioassay of Animal Hormones," by Sidney A. Thayer. Dr. Thayer first discusses the principles which should govern biological methods, i.e. the nature of the product, the determination of animal variation, the choice of a suitable standard, and units. This is followed by a short section on the statistical treatment of the data which includes a description of the standard deviation and the meaning of the regression line. This is followed by a more detailed discussion of the bioassays of the gonadotropic hormones, the remaining tropic hormones of the pituitary and the adrenal cortical hormones.—MELVIN W. GREEN.

Scientific Edition

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An *In Vitro* and *In Vivo* Study of Glass Particles in Ampules*,†

By JOHN H. BREWER and J. H. FITZGERALD DUNNING‡

This report on the effects of glass particles when *injected into animals indicates that* massive doses are required to produce damage to the organs examined during the study.

IN AN EFFORT to improve the quality of ampule preparations, studies have been made from time to time to determine the nature of contaminants found in ampules discarded after routine manufacturing inspection. It was ascertained that many of these ampules were discarded because of the presence of glass particles which range in size from less than one micron to those visible to the unaided eye. In view of the fact that many physicians are of the opinion that a glass particle injected intravenously would serve as an embolus, regardless of size, the present study has been undertaken. The investigation has also been stimulated by the development of electronic instruments which can pick up particles beyond the scope of the unaided eye. It was thought important, therefore, to determine the significance of these particles, for it has been observed that it is practically impossible to prepare ampule solutions in glass and to sterilize by autoclaving without producing some glass contamination. From a common sense viewpoint, it was believed advisable to determine at what level glass contamination in ampule solutions might be expected to be harmful. On this basis several experiments were planned which we felt might give some practical results.

Solutions containing various sizes of glass particles were prepared by the method described in the *Journal of American Public Health*, Vol. 29, p. 1147, October, 1939. These suspensions of glass particles have been injected intravenously in animals in varying amounts and over time periods extending to almost one year. These animals have been sacrificed, and chemical, histological, and pathological studies have been made. In addition, more than one thousand animals have been injected with concentrated residues obtained from ampules rejected because of particle contamination covering a period of some months of manufacture.

The object in making this study is, of

* Received July 7, 1947, from Hynson, Westcott, and Dunning, Inc., Baltimore, Md.

† This is primarily the intravenous study of this problem. A later paper will deal with carbonized particles, fiber, and intramuscular injection of these materials.

‡ The authors are indebted to Mr. A. E. Stickels for the chemical recovery of the glass particles, and to Dr. John Slaughter for the pathological sections. We also wish to thank Mr. T. J. Carski for the photomicrographs.

course, not to promote the use of ampules containing visible particles, but is an attempt to add to the rather meager information on the subject.

Medical literature abounds in discussions of silica and silicosis, the presence of silica in blood, urine and tissues, but most of this literature deals with injected or respired silica and its compounds. One is reminded of an incident in the life of Sir Harry Lauder, when he was approached at the stage door by a woman from the Salvation Army and asked to give until it hurt, replied, "Lady, the very thought of it hurts." It would seem that the very thought of injecting glass intravenously has been generally avoided in making such studies. However, in a series of papers on the biochemistry of silicic acid, King, Stantial, and Dolan, in 1933 (1) describe the intravenous administration of particulate silica in dogs. They gave 250 cc. of a SiO_2 suspension containing 0.8 mg. per cc. This was given over a period of three hours and at six hours the dog died. No information was given of particle size. Gardner and Cummings (2), in 1931, Szurek and Czaja (3) in 1933, and Simson (4, 5) in 1937 and 1940 report other experiments on the intravenous injection of particulate silica or glass. Although these papers were studies on various phases of silicosis, some of them do give particle size and their findings were helpful to us in a study of this problem. For the most part, these papers discuss the injection of particles of less than 2 microns in diameter and Simson states that 98 per cent of his particles were 0.4 micron, or less. The results of these experiments led Simson to state that at autopsy the organs and tissues of the animals showed no macroscopic evidence of disease. Microscopically, the lungs and kidneys showed numerous capillary emboli composed of aggregates of silicotic particles which later disappeared. In summarizing his experimental results, Simson stated that distinctive fibrotic lesions are produced in the liver spleen and lymph nodes of rabbits following intravenous injection of silicious dust of 0.8 micron, or less, in size.

Gardner and Cummings summarize their results as follows: Three series of rabbits were injected intravenously with 1.3 Gm., respectively, of silica particles 1 to 3 microns in diameter and silica particles 6 to 12 microns in diameter. The injections were given in divided doses and required from one to four months for their completion.

These particles were segregated in different locations according to their size. The largest ones were caught in the pulmonary capillaries, those of intermediate size in the spleen and hepatic lymph node, and the finest ones in the liver.

Fine silica particles are most active and have produced a progressive, coarsely nodular cirrhosis of the liver attended by extensive destruction of the parenchyma, followed later by regeneration in certain areas. This cirrhosis is the result of a typical, hyaline, nodular, silicotic fibrosis originating in the portal connective tissues.

Coarse silica particles, 10 to 12 microns in diameter, are much less irritating. They excite a simple foreign body type of reaction which has progressed very little in practically three years' time.

The importance of the factor of particle size in producing reaction to silica is the chief contribution of this experiment. It has been shown that large particles, 10 to 12 microns in diameter, provoke a foreign body reaction in the lungs which persists without much further change for nearly three years. Smaller ones, from 3 to 6 microns in diameter, cause slowly progressive changes in the spleen and hepatic lymph node. Very fine ones of the order of 1 micron in size result in progressive proliferation of connective tissue in the liver.

EXPERIMENTAL

In our own experimental work, we thought it important to determine what size glass particles would be encountered in ampule preparations and what size particles could be picked up by ordinary examination by a person whose vision was approximately 20–20. Using a 100-watt lamp and examining against a black and white background, the various preparations were studied. Consultation with ophthalmologists and a literature survey gave us the impression that the human eye should be able to see particles of about 50 microns under such conditions. We were surprised, however, to find that our ampule inspectors were discarding ampules containing particles no larger than 1 or 2 microns. This is

entirely understandable if one recalls having seen the reflection of an automobile windshield on a mountain so far away that the car itself could not be seen.

On examining a large number of ampules from several sources, both autoclaved and hot-air sterilized, we found most of the glass particles to be larger than 1 micron, with an average size of about 20 microns; some were as much as 75 microns in length but only a few microns in diameter. Since it is entirely possible for such particles to pass a 20-gauge needle, experimental work was planned so as to study the effect of injecting intravenously into animals suspensions containing particles in this range of size. Some animals were injected with suspensions of fine glass only; others received injections of coarser particles in both light and heavy suspension. Suspensions were made of mixtures of soft glass and of borosilicate glass, and some suspensions were made of pyrex glass only.

To describe briefly the actual technique of animal experimentation, rabbits received, by ear vein injection, varying quantities of different suspensions of glass particles. For example, one group of animals was given daily injections so that they received in thirty-two days a total of 0.416 Gm. of glass per animal. Based on body weight, this would be about 14 Gm. in man in one month, or about 0.5 Gm. per day. Since this is an absurd amount of glass to expect in ampule contamination, other animals have been injected over longer periods of time with lighter suspensions, 0.0025 Gm. per cc. for seventy-six days, and no pathology was revealed. However, an occasional particle of glass was found in the lungs.

One animal received a total of 670.5 cc. of glass suspension containing 4.156 Gm. of glass. The average daily dose was 0.0124 Gm. and the largest single injection was 0.085 Gm. This was given in 153 injections over 334 days and the animal which was seemingly well was killed and examined. In the animal which had been injected for 334 days with an average daily dose of 0.0124 Gm., macroscopically the organs revealed an enlarged liver and spleen but the other organs appeared normal. Microscopically there was a generalized picture of silicosis.

Since the smallest capillaries in the body are about 8 microns in diameter, as compared with the red cells at about 4.5 microns, we can rationalize as to what happens when glass particles as described are injected intravenously. The venous blood first encounters these small capillaries in the lungs when being aerated. As might be expected, there is some plugging of the capillaries with the larger pieces but one gets more of a Beaver Dam effect due to the sharp shape of the glass and the fluid nature of the cells; they tend to flow around the glass. When 1 cc. of a 1.3% suspension of this glass is injected daily for thirty-two days, there is no pneumonia or inflammation. The venules are dilated to capacity. The capillaries are moderately engorged. Some of the arterioles are thick walled. There is variation in the degree of aeriation atelectasis. There is poor circulation in patches. Many capillaries are bulged, widening the septiae, and these capillaries contain ragged shaped crystals, some of which are embedded in a bluish homogeneous fluid-like substance in which there are endothelial cells and leucocytes, suggesting early organization of thrombi. There are still clear spaces indicating that capillary luminae are not completely obliterated in spite of the bulging. However, there are crystals outside of the capillaries but this may be attributed to mechanical factors, since the microtome knife could not cut the particles of glass and simply shoved them along into the alveolar spaces. The smaller particles over the short time period of thirty-two days are carried along and are apparently disposed of without producing pathology, since none of the other organs are seemingly involved. Grossly the animals appeared well and had no dietary lay-off. Macroscopically the organs appeared normal and even the lungs had no gross pathology.

In the lungs of the one-year rabbit there are large discrete tubercle-like lesions which have three types of zones: (*a*) The outer lymphocytes, (*b*) Middle large cells with clear oval-shaped nuclei resembling an epitheloid pattern, and (*c*) The core is composed of polymorphonuclear leucocytes and necrotic substances. These lesions are quite large and resemble focal lesions encountered in silicosis. The capillaries are made conspicuous by small needle-shaped crystals. The alveolar septae are usually broad and practically all alveolar spaces present knob-like structures which are part of the septae bulging into alveolar space. The luminae of these circular, sharp, definite knobs are connected with the capillary wall and contain fragments of highly refractory crystalline substance. Many of these are completely occluded by large mono- and multi-nucleated cells. The multi-nucleated cells are interpreted as foreign body giant cells. There is no leucocytic infiltration into the parenchyma beyond the capillary saculations. The larger arterioles are thick walled and the capillary wall is moderately engorged and has some edema.

In the liver small glass particles are seen. The liver parenchyma is not distorted. The lobules maintain their normal pattern. The cytoplasmic cells have a coarse, flaky texture but no distinct vacuoles are seen. There is no necrosis or leucocytic reaction except in the protal triads. Here the cells consist of small lymphocytes and large multi-nucleated giant cells. The cytoplasm of these giant cells contains fine crystal and needle-like refractile substances; also, a granular matter of the same appearance. These areas of granulation are so well defined and separated from the parenchyma and septae as to be interpreted as over-stretched vessel walls. There are also multi-nucleated giant cells along the triads which cannot be localized as to inside or outside of the vessels. There are many nests of multi-nucleated giant cells; also, single giant cells scattered throughout the parenchyma, all distinctly replacing liver cords and appear to rest free in the sinusoids. All of these contain particles of refractile bodies. The striking feature of this picture is that these giant

(Photomicrographs represented in Figs. 2–6 are all of the same magnification.)

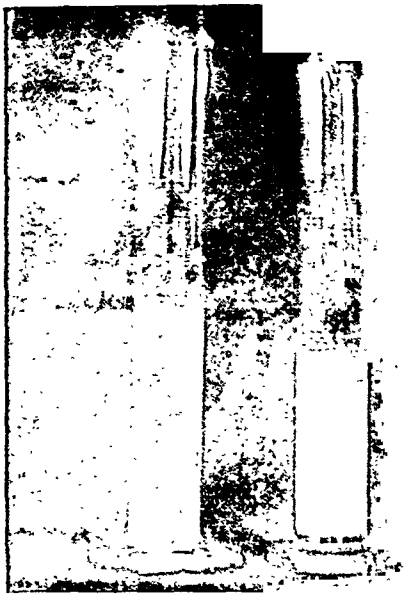


Fig. 1.—Graduate on left contains light suspension of glass. Graduate on right contains heavy suspension.

Fig. 2.—Photomicrograph of heavy glass suspension; the smallest divisions on above scale are 10 microns.

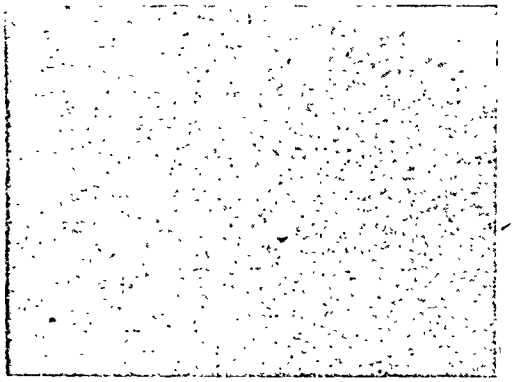


Fig. 3.—Glass particles in light suspension scale same as Fig. 2. These particles have passed through hard filter paper.

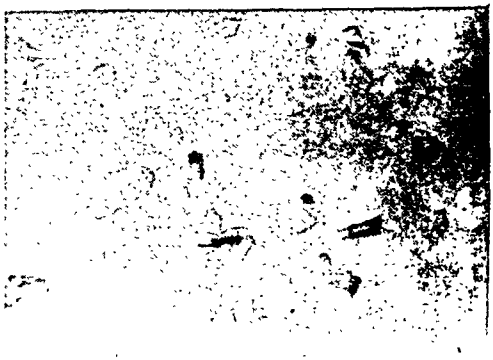
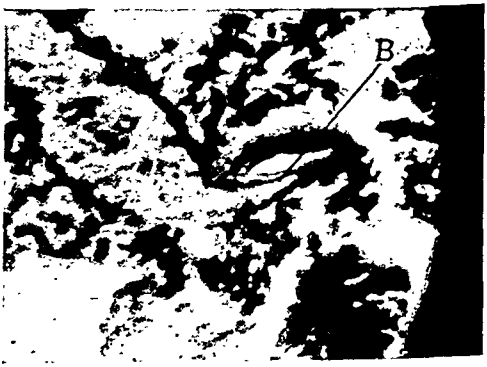


Fig. 4.—These glass particles were recovered from the lungs of a rabbit injected for 30 days with the solution shown in Fig. 2.



Figs. 5 and 6.—Show glass particles in the lungs. "A" shows that there is not a complete blocking of the capillary by the particles, and "B" shows 2 sharp glass particles lodged in the walls of a capillary.

cells are bare, thus, not producing any destruction of adjacent parenchyma nor stimulating the leucocytic zones around them. They may be Kupffler's cells. There is no cloudy swelling; the appearance may be due to some change in the feeding habit of the animal.

Spleen.—The parenchyma is studded with giant cells which have a tendency to nest and produce no reaction.

Intestine.—Fine glass particles found.

Kidney.—Occasional giant cells are found and the glomeruli are gorged with blood.

In another series of animals, in which the glass was very dilute to simulate more closely actual ampule contamination and which had been filtered to remove the larger particles, no glass was found in the lungs and no pathology was encountered. All sections looked normal and these sections have been studied by consulting pathologists who confirmed these findings.

DISCUSSION

It is interesting to note that chemical examinations were confirmatory of the histological and pathological findings in those animals which received the 1.3 per cent glass for thirty-two days. The lungs, which contained large size glass particles, were digested with acid and assay revealed that, calculated on the wet weight of lungs, there was present about 1 per cent glass. In the animal which received a lighter suspension for nearly a year almost 2 per cent glass was found. There was one very important difference. The silica in the rabbits which had received the glass for only a short time gave about the same loss with hydrofluoric acid as the control glass suspension. The animal which had had the glass for almost a year gave a 95.15 per cent loss, indicating that the body had dissolved out the basic ion, leaving silicic acid which would account for the tissue damage and generalized picture of silicosis. These findings are typical of animals injected for one year. Chemically, no glass was found in those organs in the animals which received glass over shorter periods of time except the lungs in those animals as described.

From a practical point of view, 1,089 mice were injected intravenously with the centrifugate obtained from ampules regularly processed and discarded because of particle contamination. This was continued over a period of several months to attempt to produce fatal embolism with these particles. The particles were for the most part glass, but some were charred material, probably carbon, while others were fibers. In no case did death occur, and no latent effects were noticed.

Although the industrial hygienist and those who come in constant contact with ultra small glass particles must be constantly on the alert for pathology connected with silica injection, the ampule manufacturer who meets the present requirements of producing ampules substantially free from glass particles which can be seen with the unaided eye need have no fear that his product on injection would cause a fatal embolism, even though an occasional particle falls into the solution on breaking open the ampule. Furthermore, repeated injection of occasional particles will not give rise to pathology which could be considered dangerous. We must be warned, however, against processes of manufacture such as grinding, colloidizing, or rapid temperature shock which might produce large quantities of ultra fine glass of less than 1 micron without instituting filtration procedures for removing it. Simple filtration through hard paper is not sufficient to remove such particles. Several types of filters satisfactory for bacterial removal are efficient for this purpose and should be used, although the material is subsequently sterilized by other means.

SUMMARY AND CONCLUSION

In this investigation, without attempting to be exhaustive, we have tried to prove that occasional particle contamination of ampule preparations produces no significant pathology in animals. It has been shown that truly massive doses are required to produce any pathological reaction in rabbits.

It can be reasoned, therefore, that by careful ampule processing, the dangers inherent in particle contamination can be largely eliminated.

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(5) Simson, F. W., *J. Pa* [illegible]

Microscopic-Crystallographic Properties of Crystalline Sodium Penicillin G*

By WILLIAM V. EISENBERG and GEORGE L. KEENAN

FOR THE purpose of establishing microscopic criteria that would be useful as physical data for characterizing the sodium salt of crystalline penicillin, factor G, an optical-crystallographic study was undertaken of five preparations of this substance obtained from different sources. Four samples were produced commercially while the fifth was obtained from a government research laboratory. These samples were studied by means of the immersion method as applied to the examination of crystalline material, which consists of mounting the material in oily liquids of known refractive index and making observations in ordinary light, and parallel and convergent polarized light with the polarizing microscope.

The microscopic examination of the five preparations in ordinary light at a magnification of approximately 200× revealed three crystalline habits. Elongated, fibrous rods, apparently aggregates of needle-like crystals, characterized two of these samples (Figs. 1 and 2). Two samples consisted of plates, in one case more elongated (Fig. 3) than in the other (Fig, 4), and compact radial aggregates (spherulites) represented the third habit (Fig. 5).

From a microscopic-crystallographic study of these five samples three significant refractive indices were determined, $n\alpha$ = 1.550, $n\beta$ = 1.609, $n\gamma$ = 1.620, all ±0.002.[1] The most common index for determinative purposes was found to be $n\beta$, shown on plates exhibiting partial extinction and optic axis figures. This index is most readily shown where the material crystallizes largely as plates (Fig. 3 and 4). It may also be measured on minute rods and needles occurring in the other habits (Figs. 1, 2, and 5). $n\alpha$ and $n\gamma$ are not as frequently shown as $n\beta$.

In parallel polarized light (crossed nicols) samples crystallizing in elongated fibrous rods (Figs. 1 and 2) showed parallel extinction and positive elongation. In convergent polarized light (crossed nicols), the usual interference figure shown was that of the optic axis. Platy material such as that illustrated by Fig. 3 showed that the material was biaxial negative with large axial angle as may be determined occasionally on interference figures showing curved isogyres.

Although direct examination of the salts in ordinary light showed that they exhibited various habits, this was apparently due to different methods of preparation or crystallization, and all of the samples could be converted to the same habit by recrystallizing a small amount from a drop of 95% ethyl alcohol on a microscopic slide. The material to be recrystallized was treated with the alcohol and the preparation vigorously stirred with a glass rod to induce crystallization by reseeding with the material that had not completely dissolved. The addition of *n*-butanol to the alcohol solution will aid the crystallization. This treatment of the crystalline preparations caused the substance to crystallize as plates having a 6-sided outline, many of which develop in hemimorphic formations (Fig. 6).

Heating all five of these preparations at a temperature of 100° for two hours produced no change in the crystalline habit or in the optical-crystallographic properties. It is obvious from this that crystalline penicillin sodium contains no solvent of crystallization. The five compounds of crystalline penicillin sodium studied are identical with respect to their optical-crystallographic properties and these may be used for establishing the identity of this substance.

Reproductions of photomicrographs showing the crystal structures of the samples used in this investigation are shown on the opposite page.

* Received Jan. 30, 1947, from the Microbiological Division, Food and Drug Administration, Federal Security Agency, Washington, D. C.

[1] Refractive indices are given for the D-line (Na) at 20°.

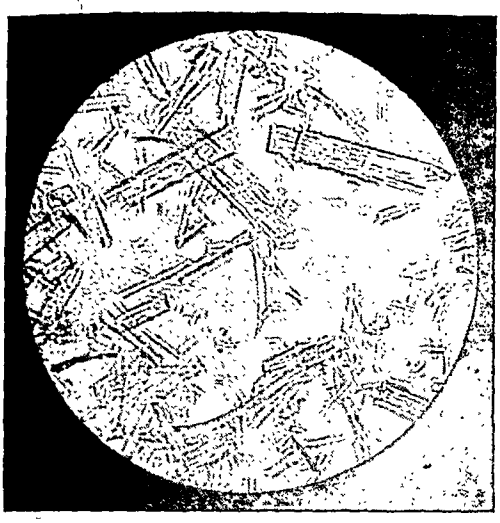


Figure 1 (150×).

Figure 2 (150×).

Figure 3 (150×).

Figure 4 (60×).

Figure 5 (150×).

Figure 6 (150×).

A Study of the Modified Knudson–Dresbach Colorimetric Evaluation of Digitalis: Extraction Studies on Digitalis*

By SAMUEL W. GOLDSTEIN†,‡

The Knudson–Dresbach colorimetric method as modified by Bell and Krantz for the assay of digitalis preparations has been applied to several samples. The results of this study are reported.

BECAUSE NONE of the present biological assay methods for digitalis is entirely satisfactory, Bell and Krantz studied the Knudson and Dresbach (1) colorimetric determination based on the Baljet (2) reaction between alkaline picrate solution and digitalis glycosides. In 1945 they published their modified method (3). This method gave results sufficiently close to the results obtained by the cat method of assay to warrant a collaborative study of the assay of digitalis and its preparations by both methods (4).

In order to check the method and its application to commercial samples of digitalis preparations available in drugstores the following study was undertaken.

Experiments were also carried out to test the necessity of the U. S. P. requirement of a twenty-four-hour period of extraction in a mechanical shaker for digitalis and preparations containing powdered digitalis.

EXPERIMENTAL

A "facile" test bottle shaker (Wagner Glass Works, New York) was used and the powdered samples and menstruum (alcohol 4 volumes, water 1 volume) were placed in oil sample bottles which fitted into the shaker cups. A Fisher Electrophotometer with green filter No. 525 and 23-cc. absorption tubes was used. All readings were made on the logarithmic scale of the instrument. The assay procedure was carried out exactly as described by Bell and Krantz (3). Readings were made at ten-minute intervals over a period of sixty minutes.

Digitalis Tinctures.—Data obtained from twenty determinations with six Reference Standard Tinctures and a number of commercial tinctures are represented graphically in Fig. 1 by plotting the readings (logarithm of the transmission) against time intervals.

The curves obtained with the U. S. P. Reference Standard Tinctures and half-strength Reference Standard Tinctures are practically identical with those reported by Bell and Krantz (3). The curves obtained with commercial tinctures assaying near or below 100% at the twenty-minute interval follow fairly closely the curves obtained with the Reference Standard Tinctures. But the curves obtained with commercial tinctures assaying considerably above 100% at the twenty-minute interval show a wide divergence from the standard curves. The curve showing the highest values was obtained from results with two samples from manufacturer No. 5, and one other sample. The drugstore operator could not estimate the age of this latter preparation, and it might be possible that he assigned it to the wrong manufacturer. The next highest curve represents another sample from manufacturer No. 5.

Bell and Krantz (5), as a result of their studies with the digitalis glycosides, have assumed that the intensity of the Baljet reaction is proportional to the amount of glycoside (lactone) present, but that the degree of isomerization [which occurs in the presence of the Baljet reagent] is not directly proportional to the concentration of the glycoside. They conclude that if the observed intensity of the Baljet reaction is regarded as the resultant of these two factors, and since one of them does not show direct proportionality to the concentration, the resultant likewise will not. Their assumptions and conclusions are definitely applicable to the results obtained with most of the tinctures of widely varying potencies as shown in Fig. 1.

Digitalis Tablets.—Tinctures were prepared from eleven commercial digitalis tablets using the U. S. P. XIII procedure for the assay of Digitalis Tablets. These tinctures were tested in the same manner as the commercial tinctures, and the results are given graphically in Fig. 2. The curves obtained follow the same general pattern as observed with the commercial tinctures, with two exceptions. These samples show an unusual divergence from the general curve and undoubtedly contained some substance, possibly lactose, which gives a strongly increasing color reaction with the Baljet reagent.

The results from both the tinctures and the tablets show that where there is evident divergence from the standard curves, this divergence increases as the time interval increases. It may be assumed

* Received March 15, 1947, from the State of Maryland Department of Health, Baltimore.
† Pharmaceutical Chemist, State of Maryland Department of Health.
‡ The author is indebted to Mr. J. R. McComas for assistance with the charts.

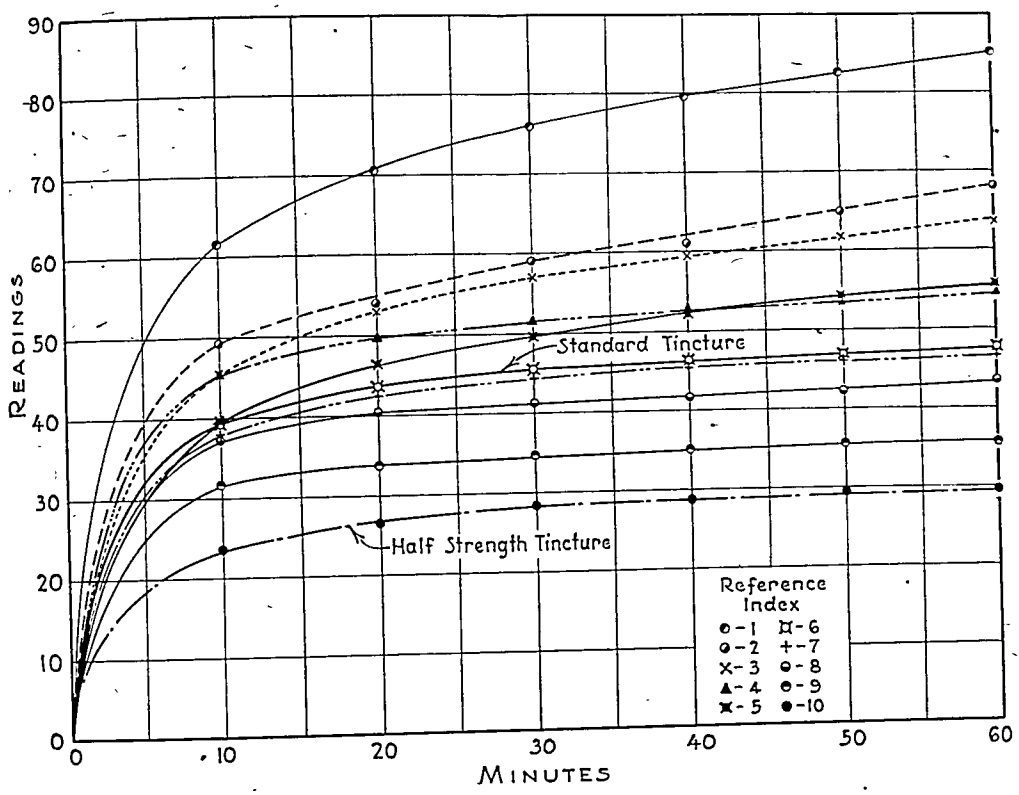


Fig. 1.—Reference Standard and Commercial Tinctures

Curve No. 1. Average values for samples 17283 S, 17287 S, and 8922 E, which assayed 180% ± 4 at 20 minutes.
Curve No. 2. Sample 17293 S, which assayed 128% at 20 minutes.
Curve No. 3. Average values for samples 17278 S and 17315 S, which assayed 130% ± 3 at 20 minutes.
Curve No. 4. Average values for samples 17280 S, 17292 S, and 8925 E, which assayed 117% ± 2 at 20 minutes.
Curve No. 5. Average values for samples 17279 S, 17286 S, and 17288 S, which assayed 109% ± 3 at 20 minutes.
Curve No. 6. Reference Standard Tincture.
Curve No. 7. Average values for samples 17276 S, 17281 S, and 17282 S, which assayed 100% ± 2 at 20 minutes.
Curve No. 8. Average values for samples 17289 S, 17290 S, 8923 E, and 8924 E, which assayed 90% ± 2 at 20 minutes.
Curve No. 9. Sample 17291 S, which assayed 68% at 20 minutes.
Curve No. 10. Half-strength Reference Standard Tincture.

that the earliest consistently reproducible reading would be more likely to give results based on differences in concentration of the glycosides in *D. purpurea* and its preparations. This happens to be the reading taken about twenty minutes after the addition of the reagent to the prepared sample. Bell and Krantz have selected this time interval. Published results (3, 5, 6) indicate that values obtained by the modified Knudson–Dresbach chemical assay are generally higher than the values obtained by bioassay procedures. These differences would be further accentuated if values were based on readings taken after an interval longer than twenty minutes, especially with preparations considerably stronger than the standard tincture. This purely factual consideration is sufficient to warrant the selection of the twenty-minute reaction period.

The assay results for the commercial tinctures at the different time intervals, together with the approximate length of time the tinctures were known to have been in the drugstores, are given in Table II. It is noteworthy that of the identified tinctures the highest percentages were obtained with old products of manufacturer No. 5. The comparatively fresh product of the same manufacturer showed an increase of 33% between the twenty-minute and sixty-minute determinations. The next greatest increase in percentage between the twenty-minute and sixty-minute determinations is 18%. Apparently some substance is present in the No. 5 preparations (possibly introduced in the menstruum) which slowly changes or causes a change under ordinary conditions with the change being accelerated in the presence of the alkaline picrate reagent. Tinctures

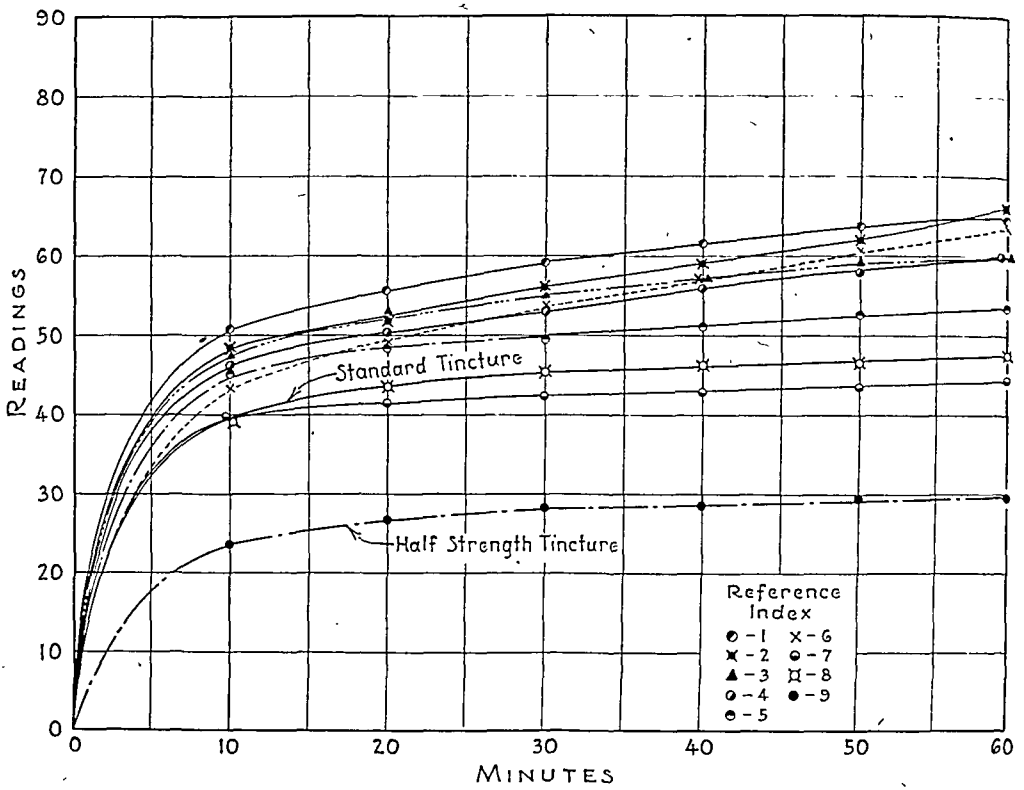


Fig. 2.—Reference Standard Tinctures and Tinctures Prepared from Digitalis Tablets

Curve No. 1. Average values for samples 17256 S and 17265 S, which assayed 135% ± 2 at 20 minutes.
Curve No. 2. Sample 17260 S, which assayed 124% at 20 minutes.
Curve No. 3. Average values for samples 17264 S and 17266 S, which assayed 128% at 20 minutes.
Curve No. 4. Sample 17259 S, which assayed 122% at 20 minutes.
Curve No. 5. Sample 17261 S, which assayed 113% at 20 minutes.
Curve No. 6. Sample 17258 S, which assayed 116% at 20 minutes.
Curve No. 7. Average values for samples 17257 S, 17262 S, and 17263 S, which assayed 95% ± 5 at 20 minutes.
Curve No. 8. Reference Standard Tincture.
Curve No. 9. Half-strength Reference Standard Tincture.

giving such results should be checked by biological assay.

Omitting from consideration the established products of manufacturer No. 5, fifteen of the twenty-three tinctures fall within the limits allowed by the U. S. P. The age of sample 17283 S could not be established, and it appears doubtful that it was assigned to the correct manufacturer.

Of the eleven samples of Digitalis Tablets tested, four samples exceeded the U. S. P. limit in potency. However the amounts by which the upper limit was exceeded were 2%, 2%, 7%, and 10%. These four samples were products of the same manufacturer, and their curves indicate the absence of any interfering substance.

Extraction Studies.—Digitalis tinctures were prepared from U. S. P. Standard Reference Powder by the official procedure except that eight-, sixteen-, and twenty-four hour extraction periods in a mechanical shaker were used. The averages of values for three, twelve, and five series of readings of the respective products are given in Table I. The values obtained indicate that for all practical purposes an extraction period of eight hours in a mechanical shaker allows complete extraction of the glycosides of *D. purpurea*.

TABLE I.—LOGARITHMIC SCALE READINGS FOR TINCTURES PREPARED FROM REFERENCE STANDARD DIGITALIS USING DIFFERENT EXTRACTION PERIODS

Reading Time, Min.	8-Hr. S[a]	8-Hr. S/2[b]	16-Hr. S	16-Hr. S/2	24-Hr. S	24-Hr. S/2
10	39.8	23.5	39.3	23.5	40.0	23.8
20	43.7	26.5	43.3	26.7	43.9	27.1
30	45.3	27.5	45.3	28.0	46.0	28.6
40	46.0	28.0	46.0	28.7	46.5	29.3
50	46.5	28.5	46.6	29.1	47.2	29.6
60	47.0	28.7	47.1	29.3	47.3	30.1

[a] S is the full-strength Reference Standard Tincture.
[b] S/2 is the half-strength Reference Standard Tincture.

Tinctures were prepared from commercial Digitalis Tablets by the U. S. P. XIII procedure and also

by limiting the extraction period to eight hours. The results of the assays of the tinctures obtained by both procedures are given in Table III. These results appear to establish beyond a doubt that an eight-hour extraction period in a mechanical shaker is sufficient for complete extraction of the digitalis glycosides.

Discussion of Analytical Method.—In its present state the Knudson-Dresbach method as modified by Bell and Krantz for the evaluation of digitalis and its preparations may not appear to satisfy all the requirements for an official chemical assay. But neither is the official biological method entirely satisfactory. The chemical procedure does yield results

TABLE II.—PER CENT POTENCY IN TERMS OF STANDARD OF COMMERCIAL DIGITALIS TINCTURES

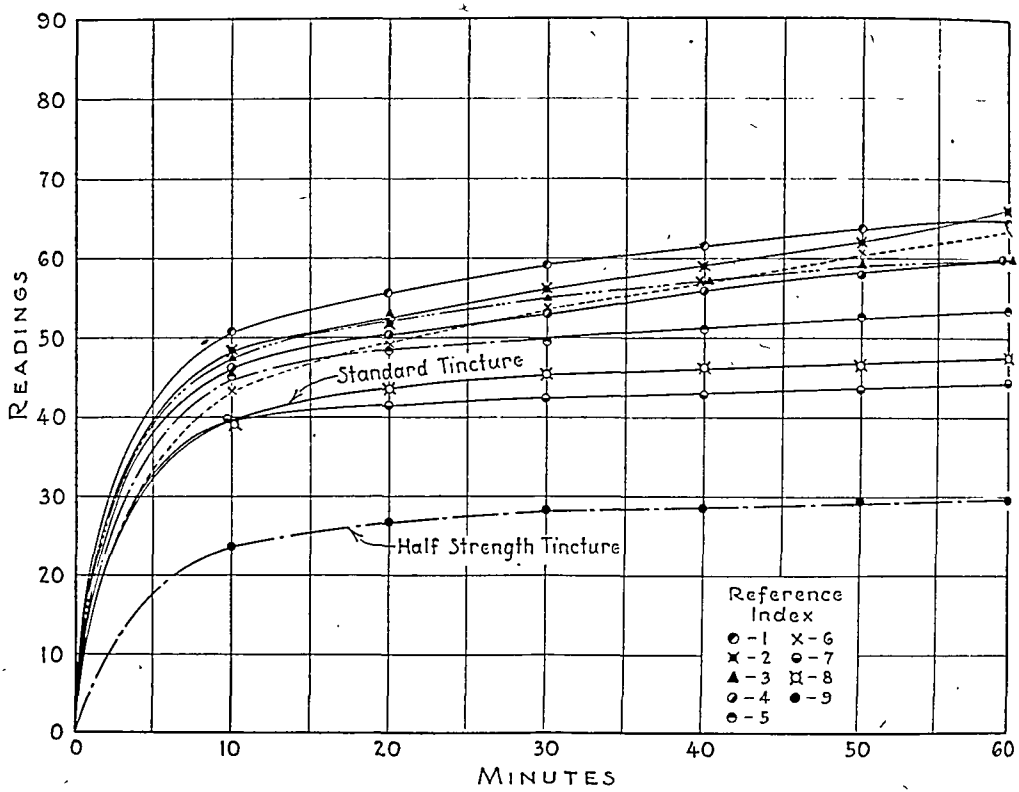
Sample No.	Mfr. No.	Time in Store	% at 10 Min.	% at 20 Min.	% at 30 Min.	% at 40 Min.	% at 50 Min.	% at 60 Min.
17273 S	1	1 wk.	...	95	...	...	...	...
17274 S	2	3 mo.	...	126	...	...	...	...
17275 S	1	5 mo.	...	97	...	...	...	...
17276 S	3	2 yr.	...	102	100	100	100	100
17277 S	3	1 yr.	...	120	123	124	126	126
17278 S	..	....	...	132	134	141	143	146
17279 S	3	2 mo.	...	111	110	113	117	121
17280 S	4	6 mo.	120	115	111	111	113	113
17281 S	..	4 mo.	...	102	100	100	100	100
17282 S	1	2 mo.	100	98	96	96	96	96
17283 S	3	....	161	181	187	196	197	207
17284 S	4	2 yr.	139	143	141	144	147	149
17285 S	2	2 mo.	121	138	141	147	150	154
17286 S	2	10 mo.	102	106	114	120	123	124
17287 S	5	18 mo.	173	178	186	195	194	199
17288 S	2	1 yr.	103	108	114	120	120	121
17289 S	1	1 mo.	90	90	89	87	87	89
17290 S	1	....	94	91	89	86	86	87
17291 S	1	4 mo.	72	68	67	65	65	64
17292 S	2	6 mo.	106	118	125	127	128	129
17293 S	5	2 mo.	127	130	140	144	153	163
17315 S	2	2 mo.	116	127	133	136	141	143
8922 E	5	7 mo.	182	184	191	200	207	210
8923 E	..	7 mo.	97	92	90	91	90	89
8924 E	1	2 wk.	93	88	85	85	84	83
8925 E	4	6 mo.	121	119	114	116	117	114

TABLE III.—PER CENT POTENCY IN TERMS OF STANDARD DIGITALIS TABLETS

Samples Prepared by Shaking for 8-Hour and for 24-Hour Periods

Sample No.		Instrument Reading Intervals in Minutes 10	20	30	40	50	60
17256 S,[a]	8 hr.	139	139	141	149	153	154
	24 hr.	133	133	139	143	150	151
17257 S,	8 hr.	95	91	88	90	90	90
	24 hr.	95	89	88	91	91	93
17258 S,	8 hr.	110	114	122	128	138	143
	24 hr.	113	117	124	134	141	146
17259 S,	8 hr.	126	124	126	133	137	140
	24 hr.	121	120	120	127	131	135
17260 S,[b]	8 hr.	127	124	130	137	144	151
	24 hr.	125	124	130	137	144	151
17261 S,	8 hr.	120	112	110	115	116	115
	24 hr.	120	114	112	115	119	118
17262 S,[c]	8 hr.	113	100	96	97	99	99
	24 hr.	109	100	96	94	96	97
17263 S,	8 hr.	98	94	91	88	87	87
	24 hr.	102	97	93	90	90	90
17264 S,[a]	8 hr.	127	128	132	134	139	140
	24 hr.	127	128	132	134	139	140
17265 S,[a]	8 hr.	140	133	136	142	146	147
	24 hr.	137	133	136	142	146	147
17266 S,[a]	8 hr.	124	126	126	130	131	130
	24 hr.	132	129	131	136	140	138

[a] Samples, all from the same manufacturer, yielded green filtrates.
[b] Green colored, coated tablets yielded green filtrates.
[c] The 8-hour preparation was tested 5 days earlier than the 24-hour preparation. The prevailing room temperatures were 21° and 19.5°, respectively.



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20	43.7	26.5	43.3	26.7	43.9	27.1
30	45.3	27.5	45.3	28.0	46.0	28.6
40	46.0	28.0	46.0	28.7	46.5	29.3
50	46.5	28.5	46.6	29.1	47.2	29.6
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TABLE II.—PER CENT POTENCY IN TERMS OF STANDARD OF COMMERCIAL DIGITALIS TINCTURES

Sample No.	Mfr. No.	Time in Store	% at 10 Min.	% at 20 Min.	% at 30 Min.	% at 40 Min.	% at 50 Min.	% at 60 Min.
17273 S	1	1 wk.	...	95	...	...	...	...
17274 S	2	3 mo.	...	126	...	...	...	...
17275 S	1	5 mo.	...	97	...	...	...	...
17276 S	3	2 yr.	...	102	100	100	100	100
17277 S	3	1 yr.	...	120	123	124	126	126
17278 S	..	....	...	132	134	141	143	146
17279 S	3	2 mo.	...	111	110	113	117	121
17280 S	4	6 mo.	120	115	111	111	113	113
17281 S	..	4 mo.	...	102	100	100	100	100
17282 S	1	2 mo.	100	98	96	96	96	96
17283 S	3	....	161	181	187	196	197	207
17284 S	4	2 yr.	139	143	141	144	147	149
17285 S	2	2 mo.	121	138	141	147	150	154
17286 S	2	10 mo.	102	106	114	120	123	124
17287 S	5	18 mo.	173	178	186	195	194	199
17288 S	2	1 yr.	103	108	114	120	120	121
17289 S	1	1 mo.	90	90	89	87	87	89
17290 S	1	....	94	91	89	86	86	87
17291 S	1	4 mo.	72	68	67	65	65	64
17292 S	2	6 mo.	106	118	125	127	128	129
17293 S	5	2 mo.	127	130	140	144	153	163
17315 S	2	2 mo.	116	127	133	136	141	143
8922 E	5	7 mo.	182	184	191	200	207	210
8923 E	..	7 mo.	97	92	90	91	90	89
8924 E	1	2 wk.	93	88	85	85	84	83
8925 E	4	6 mo.	121	119	114	116	117	114

TABLE III.—PER CENT POTENCY IN TERMS OF STANDARD DIGITALIS TABLETS

Samples Prepared by Shaking for 8-Hour and for 24-Hour Periods

Sample No.		Instrument Reading Intervals in Minutes 10	20	30	40	50	60
17256 S,[a]	8 hr.	139	139	141	149	153	154
	24 hr.	133	133	139	143	150	151
17257 S,	8 hr.	95	91	88	90	90	90
	24 hr.	95	89	88	91	91	93
17258 S,	8 hr.	110	114	122	128	138	143
	24 hr.	113	117	124	134	141	146
17259 S,	8 hr.	126	124	126	133	137	140
	24 hr.	121	120	120	127	131	135
17260 S,[b]	8 hr.	127	124	130	137	144	151
	24 hr.	125	124	130	137	144	151
17261 S,	8 hr.	120	112	110	115	116	115
	24 hr.	120	114	112	115	119	118
17262 S,[c]	8 hr.	113	100	96	97	99	99
	24 hr.	109	100	96	94	96	97
17263 S,	8 hr.	98	94	91	88	87	87
	24 hr.	102	97	93	90	90	90
17264 S,[a]	8 hr.	127	128	132	134	139	140
	24 hr.	127	128	132	134	139	140
17265 S,[a]	8 hr.	140	133	136	142	146	147
	24 hr.	137	133	136	142	146	147
17266 S,[a]	8 hr.	124	126	126	130	131	130
	24 hr.	132	129	131	136	140	138

[a] Samples, all from the same manufacturer, yielded green filtrates.
[b] Green colored, coated tablets yielded green filtrates.
[c] The 8-hour preparation was tested 5 days earlier than the 24-hour preparation. The prevailing room temperatures were 21° and 19.5°, respectively.

that check very well, and earlier studies (4) have indicated that both methods are measuring the same activity of the drug. The chemical procedure certainly can be useful in control laboratories that are not equipped for biological procedures. As a precaution, the final readings can be continued at ten-minute intervals beyond the critical twenty-minute period, and the curve, obtained by plotting the readings against time, can be compared with curves prepared by assaying biologically standardized samples. When a curve is definitely out of line, indicating the presence of an interfering substance, that sample can be sent to another laboratory for checking by a biological procedure.

Inspector H. Bernhardt collected the commercial samples from drugstores and obtained information, wherever possible, as to the length of time the tinctures had been in stock. This did not take into account the age of the preparations at the time the pharmacists purchased them. Comparisons under these conditions show that aging of properly prepared Digitalis Tincture has no apparent effect on the course of the chemical assay, and that high or low results cannot be attributed to this factor.

Daily room temperatures were recorded and these varied from 19.5° to 25.5°. These variations appear to have some effect on the rate of the reaction, but they apparently affect the standard and sample in the same manner and can be discounted.

Five of the eleven Digitalis Tablets yielded tinctures which gave green filtrates in the course of the assay. Four of these tablets were products of the same manufacturer and the green color appeared to have no influence on the course of the reaction. The other product was a green-colored, coated tablet, and its tincture gave an accelerated reaction. This acceleration cannot be attributed to the green color, but rather to the constituents of the coating material.

Discussion of Extraction Studies.—Our fifteen-year-old shaker is very efficient while running, but it demands attention at the most unexpected times. Consequently the agitated extractions were carried out in eight-hour periods. The extractions requiring more than eight hours were interrupted by sixteen-hour periods (overnight) in a refrigerator at 2°. Thus, while with the shortest extraction period the drug was in contact with the menstruum exactly eight hours, with the sixteen-hour and twenty-four-hour preparations the drug was actually in contact with the menstruum thirty-two hours and fifty-six hours, respectively. This arrangement obviously favored the longer extraction periods, but, nevertheless, the eight-hour extraction period in a mechanical shaker appears to have established itself beyond doubt as a period of sufficient length for practically complete removal of the digitalis principles. In order to be doubly certain, the drug and menstruum could be mixed, allowed to macerate overnight, and then be subjected to an eight-hour period in a mechanical shaker.

SUMMARY

1. The Knudson-Dresbach method as modified by Bell and Krantz for the colorimetric determination of digitalis activity has been studied.

2. The use of this method in control laboratories not equipped for biological assay work has been discussed, and is recommended.

3. Extractions of Powdered Digitalis and Digitalis Tablets by the U. S. P. XIII procedure, except for variations in the length of the extraction period, have proved conclusively that under the described conditions an eight-hour extraction in a mechanical shaker will remove practically all of the active digitalis principles soluble in the official menstruum.

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Chemical Differentiation of Desoxyephedrine from Other Sympathomimetic Amines*

By THOMAS J. HALEY†

Methods for the chemical identification and differentiation of isomers of desoxyephedrine from each other and from twelve additional sympathomimetic amines are described. Of the reagents employed, chloroauric, chloroplatinic, and picric acids were most useful since they produced crystalline precipitates having definite melting points.

THE PRESENT commercial availability of potent drugs such as desoxyephedrine and related sympathomimetic amines makes it imperative that an easy method for their identification be made available. Thus far only two cases of toxicity from prolonged use of desoxyephedrine have been reported (2, 4).

Dultz (5) has shown that the drug may be distilled from alkalized cadaveric material and the quantity therein determined by titration. Urinary excretion of desoxyephedrine as well as other similar amines has been determined by Richter (11) with a nonspecific picric acid reagent. Both of the above methods may be used to determine the amount of drug causing death but they will not differentiate between its various forms or between it and other similar amines. Compounds of this type are usually identified by converting them into known derivatives. Of the three forms of desoxyephedrine only the *d*-isomer has been investigated thoroughly in this manner. All reported derivatives of desoxyephedrine are given in Table I. Table II shows the structural differences and the melting points of all the sympathomimetic amine salts studied in this investigation.

This study of the reaction between fifteen sympathomimetic amine salts and ten alkaloidal reagents was undertaken in an attempt to find a rapid method for the identification and differentiation not only of the three isomers of desoxyephedrine but also of the other compounds listed.

TABLE I.—RECORDED DERIVATIVES OF DESOXYEPHEDRINE

Isomer	Derivative	Melting Point, °C.	Reference
Dextro	Hydrochloride	170–171	Ogata (10)
		171–173	Novelli and Tainter (9)
		171–173	Rosenmund, *et al.* (12)
	Bitartrate	118–119	Ogata (10)
	Picrate	144–145	Ogata (10)
	Mercuric Chloride	152–153	Ogata (10)
	Gold Chloride	127	Ogata (10)
	Platinic Chloride	214–215	Ogata (10)
	Picrolonate	183	Dultz (5)
Racemic	Hydrochloride	133–135	Novelli (8)
		132–133	Novelli and Tainter (9)
		134	Rosenmund, *et al.* (12)
	Platinic Chloride	198–199	Erlenmeyer and Simon (6)
	Thiourea	134	Erlenmeyer and Simon (6)
	Sulfathiazole	118–120	Hamilton, *et al.* (7)
	Sulfadiazine	187–189	Hamilton, *et al.* (7)
Levo	Tartrate	159–160	Novelli and Tainter (9)

EXPERIMENTAL

All the compounds tested were made up in one per cent solutions; the alkaloidal reagents were prepared as directed by Autenrieth and Bauer (1), and the reagents for the Copper Sulfate test according to the directions in the United States Pharmacopœia (13). One cubic centimeter of the amine solution to be tested was placed in a small test tube and

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† The author wishes to thank Dr. K. K. Chen of Eli Lilly and Co. for the *l*-ephedrine sulfate and the Tuamine Sulfate, Dr. H. C. Longnecker of Smith, Kline and French Laboratories for the Benzedrine and Dexedrine Sulfates and the Paredrine Hydrobromide, Dr. G. R. Hazel of Abbott Laboratories for the *d*- and *l*-desoxyephedrines, Dr. R. S. Shelton of Wm. S. Merrell Co. for the Vonedrine and Nethamine Hydrochlorides, Mr. Louis Roeg of Merck and Co. for the *dl*-ephedrine hydrochloride and Dr. M. L. Moore of Frederick Stearns and Co. for the Synephrine Tartrate, and the *d*-, *l*- and *dl*-Neosynephrines used in this study.

TABLE II.—STRUCTURAL DIFFERENCES OF THE AMINES TESTED

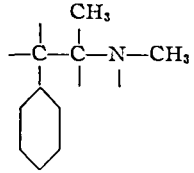
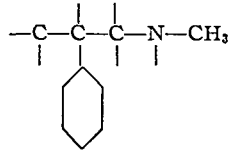
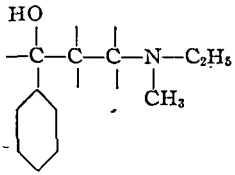
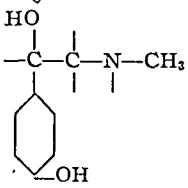
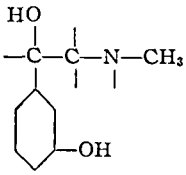
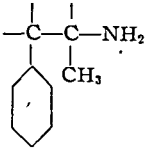
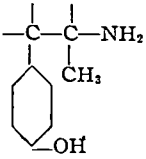
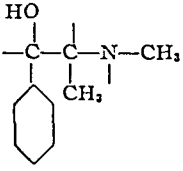
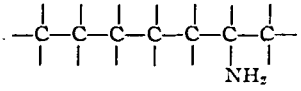
Amine	Structural Formula	Melting Point of Salts
Desoxyephedrine (1-phenyl-2-methylaminopropane)		*d*-Hydrochloride 170–173° *dl*-Hydrochloride 132–135° *l*-Tartrate 159–160°
Vonedrine (2-phenyl-1-methylaminopropane)		*dl*-Hydrochloride 144–148°
Nethamine (1-phenyl-3-methylethylamino-1-propanol)		*dl*-Hydrochloride 186°
Synephrine (Sympatol) (1-(*p*-hydroxyphenyl)-2-methylaminoethanol)		*dl*-Tartrate 183–185°
Neo-Synephrine (1-(*m*-hydroxyphenyl)-2-methylaminoethanol)		*d*-Tartrate 98–100° *dl*-Hydrochloride 142–143° *l*-Hydrochloride 141.5–143°
Benzedrine (1-phenyl-2-aminopropane)		*dl*-Sulfate above 300° *d*-Sulfate above 300° (Dexedrine)
Paredrine (1-(*p*-hydroxyphenyl)-2-aminopropane)		Hydrobromide 189–190°
Ephedrine (1-phenyl-2-methylamino-1-propanol)		*dl*-Hydrochloride 187–188° *l*-Sulfate 240–247.5°
Tuamine (2-aminoheptane)		Sulfate 275–277°

TABLE III—REACTIONS BETWEEN ALKALOIDAL REAGENTS AND SYMPATHOMIMETIC AMINES

Amine	U. S. P. $CuSO_4$ Test	Chloro-auric Acid, 1:20	Chloro-platinic Acid, 5%	Silico-tungstic Acid, 5%	Phospho-molybdic Acid, 10%	Phospho-tungstic Acid	Mayer's Reagent	Mercuric Chloride, 1:20	Iodo-Potassium Iodide Solution (Wagner's Reagent)	Saturated Aqueous Solution of Picric Acid
-Ephedrine Sulfate	Purple ether layer	Negative	Negative	Amorphous white precipitate	Amorphous yellow precipitate	Negative	Slight precipitate on standing	Negative	Amorphous brown precipitate	Negative
dl-Ephedrine Hydrochloride	Purple ether layer	Negative	Negative	Amorphous white precipitate	Amorphous yellow precipitate	Negative	Negative	Negative	Negative	Negative
dl-Desoxyephedrine Hydrochloride	Negative	Yellow needles 115°	Orange needles 199°	Amorphous white precipitate	Amorphous yellow precipitate	Amorphous white precipitate 186–195°	Amorphous yellow precipitate	Colorless crystals on standing 126°	Amorphous black precipitate	Yellow needles 118°
d-Desoxyephedrine Hydrochloride	Negative	Yellow needles 130°	Orange needles 212°	Amorphous white precipitate	Amorphous yellow precipitate	Amorphous white precipitate 206–216° d.	Amorphous yellow precipitate	Colorless crystals on standing 156°	Amorphous brown precipitate	Yellow needles 146°
-Desoxyephedrine Tartrate	Negative	Yellow needles 126°	Orange needles 215° d.	Amorphous white precipitate	Amorphous yellow precipitate	Amorphous white precipitate	Amorphous yellow precipitate	Negative	Amorphous brown precipitate	Yellow needles 144°
Vonedrine Hydrochloride	Negative precipitate reduced	Yellow needles 158°	Orange needles on standing 200°	Amorphous white precipitate	Amorphous yellow precipitate	Negative	Amorphous yellow precipitate	Colorless crystals on standing 168°	Amorphous brown precipitate	Yellow needles on standing 153°
Nethamine Hydrochloride	Negative	Yellow needles on standing 109°	Negative	Amorphous white precipitate 145–150° d.	Amorphous yellow precipitate	Negative	Amorphous yellow precipitate	Negative	Amorphous reddish-brown precipitate	Amorphous yellow precipitate
dl-Benzedrine Sulfate	Negative	Negative	Negative	Amorphous white precipitate 209–220° d.	Amorphous yellow precipitate	Amorphous white precipitate	Negative	Negative	Negative	Negative
d-Benzedrine (Dexedrine Sulfate	Negative	Negative	Negative	Amorphous white precipitate 210–216° d.	Amorphous yellow precipitate	Amorphous white precipitate	Negative	Negative	Negative	Negative
Paredrine Hydrobromide	Negative	Solution turned blood-red	Negative	Negative	Brown crystals on standing 190–210° d.	Negative	Negative	Negative	Negative	Negative
Synephrine Tartrate	Negative	Negative	Negative	Negative	Amorphous yellow precipitate immediately soluble	Negative	Negative	Negative	Negative	Negative
dl-Neo-Synephrine Hydrochloride	Purple Ether layer	Negative	Negative	Negative	Amorphous brown-yellow precipitate	Negative	Negative	Negative	Negative	Negative
l-Neo-Synephrine Hydrochloride	Purple Ether layer	Negative	Negative	Negative	Amorphous brown-yellow precipitate	Negative	Negative	Negative	Negative	Negative
d-Neo-Synephrine Tartrate	Negative	Negative	Negative	Negative	Amorphous yellow precipitate immediately soluble	Negative	Negative	Negative	Negative	Negative
Tuamine Sulfate	Negative precipitate reduced	Yellow needles on standing 80°	Negative	Amorphous white precipitate	Amorphous yellow precipitate	Amorphous white precipitate 225–250°.	Negative	Negative	Negative	Yellow needles 100°

drops of the reagent added. Observations were made immediately and after one, two, three, four, and twenty-four hours. The results with all the amines tested are given in Table III. Where crystalline or amorphous precipitates were formed, they were filtered, placed in a vacuum desiccator over P_2O_5 two hours, and their melting points then determined using a Thiele tube. All melting points reported are uncorrected.

DISCUSSION

Chen (3) investigating the $CuSO_4$-NaOH test on a series of twenty-seven aromatic amines, including six optical isomers of ephedrine, found that those having an OH group on the second C atom from the amine group gave a positive test. By comparison of Tables II and III it may be seen that both the *l*- and *dl*-ephedrines and *l*- and *dl*-Neosynephrines gave a positive purple coloration which, in the case of the latter, was not ether soluble. The negative test given by *d*-Neosynephrine, as well as Synephrine, may have been due to the use of their tartaric acid salts. This acid probably interferes by preferentially reacting with the alkaline copper sulfate as it does in Fehling's solution. If inorganic acid salts were employed, both *d*-Neosynephrine and Synephrine would give a positive test. The results obtained with the other amines were to be expected because they did not have the desired group.

The results obtained with phosphomolybdic and silicotungstic acids were similar to those described for other compounds by Autenrieth and Bauer (1). In those instances where precipitates were formed, they were generally so unstable that they could not be isolated before decomposition. Also, the fact that all the precipitates were the same color prevents the use of this test for identification purposes. The phosphotungstates isolated had no definite melting points and thus were of little value in identifying the amines.

All isomers of desoxyephedrine, as well as both Vonedrine and Nethamine, reacted with most of the reagents employed, and it was only by the use of melting point differences that these drugs could be identified. The crystalline precipitates formed with chloroauric and chloroplatinic acids appeared immediately, could be filtered and dried rapidly and form the basis of a rapid method of identification and differentiation. The other crystalline precipitates which formed upon standing would add further evidence as to the exact amine causing the toxic symptoms or death.

Where "Negative" appears in Table III, it should not be concluded that no reaction occurred with the reagent, since the derivatives may have been formed but were too soluble to precipitate out of solution. The failure of *l*-desoxyephedrine to give a precipitate with mercuric chloride must be ascribed to persistent supersaturation of the system in the absence of the solid phase rather than to the greater solubility of the "*l*"-isomer derivative.

SUMMARY

1. A method has been described for the chemical identification and differentiation of the isomers of desoxyephedrine from each other and from twelve other sympathomimetic amines.

2. Four new derivatives of *dl*-desoxyephedrine and three new derivatives of *l*-desoxyephedrine have been described.

3. Chloroauric, chloroplatinic, and picric acids were the most useful of the reagents employed with the sympathomimetic amines because they gave crystalline precipitates having definite melting points. Further, they form these precipitates quickly and thus enable a rapid identification to be made.

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A Graphical Calculator for Bioassays*

By FRANK M. GOYAN and J. DUFRENOY †

A new graphical calculator built as a two-dimensional slide rule has been designed to expedite the plotting and reading of reference curves for bioassays. This device has been applied to the assay of penicillin using logarithmic scales for both the concentrations of pencillin and the diameters of the zones of inhibition.

PENICILLIN ASSAY by the standard cup-plate method is accomplished by establishing the relationship between zone size (in mm.) and concentration of penicillin in Oxford units per cc. (u./cc.). Several investigators have described graphical methods of computing concentration from zone size based upon simple arithmetic functions (1–3). So-called penicillin slide rules now in common use are based upon this principle (4).

In an attempt to obtain a linear function E. J. de Beer, M. B. Sherwood (5), L. F. Knudsen, W. A. Randall (6), and C. I. Bliss (7) substituted log concentration for concentration. Knudsen and Randall stressed that "It has been shown repeatedly at several laboratories that, for doses of penicillin equivalent to from 0.25 unit per ml. to 3 units per ml. in each cup, there is a straight line relationship between the diameter of the zone of inhibition and the logarithm of the dose. In other words, if the log of the dose is plotted against the diameter of the zone of inhibition, the points indicate a straight line." Assuming this straight line relationship to be valid, determination of potencies depends mainly on the determination of the slope of the line for two known dilutions of the standard and two corresponding dilutions of the unknown. To that end Knudsen and Randall (6) published a chart for determining potency as per cent of standard from the two-concentration plate method and Bliss (7) published charts for penicillin assay based on two test solutions for both the standard and the unknown. One of us in connection with the study of a great number of routine penicillin assays, found it advantageous to plot log concentrations against log diameters: the "standard" curve is then represented by two straight lines, one sloping more steeply in the range from 0.5 u./cc. to 1.5 u./cc., and the other sloping less steeply from 1.5 u./cc. to 4 u./cc.[1] Excellent agreement was obtained between concentrations read by standard methods (using a reference curve plotted on arithmetic paper) and those read from the logarithmic plot.

Reading the zones from plates submitted to the "physical development" previously described (8) made it possible to graph the "log concentration-log diameter" reference curve as a straight line throughout the range from 1 to 8 u./cc. However, the use of commercially available logarithmic paper entails various difficulties. To remove these difficulties one of us devised a two-dimensional slide rule which performs the mathematical function of a special logarithmic paper, with the additional advantage (among others) that the dilution factors can be read directly (see scale *B* of Fig. 1) and the standard zone sizes set up without redrawing the curve, simply by moving the panel *P* up or down in relation to the ordinate scale *O*.

DESCRIPTION OF APPARATUS

The graphical calculator or two-dimensional slide rule shown in Fig. 1 was constructed as follows:

A 14" × 12" piece of plywood (*F*) is used as the support of the frame; the basal part of the frame consists of a slide rule, made by screwing on the 14" × 12" piece of plywood two pieces of wood (*G*) which are beveled so as to form the groove in which the beveled ruler (*B*) slides freely; the sides of the frame (*F*) are made up each of a piece of beveled plywood, allowing a beveled panel (*P*) (also of plywood) to slide up and down.

The ruler (*O*), bearing the ordinate scale, is secured to the ruler (*B*) by means of a T-shaped piece of brass which is built up to serve as a handle for

* [illegible] from the University of California, San Francisco, Calif.
† [illegible] made to Cutter Laboratories, [illegible] grant in support of this research, and to Prof. Robertson Pratt for help in the preparation of the manuscript.

[1] For instance, in actual tests the slope was calculated as (log 23.5 − log 18.09)/(log 1.5 − log 0.5) = 0.24 for the steeper straight line in the range from 0.5 to 1.5 u./cc. and as (log 27.5 − log 23.5)/(log 4 − log 1.5) = 0.15 for the less steep straight line in the range from 1.5 to 4 u./cc.

sliding ruler (*B*) and attached ordinate scale. To the basal part of the frame is then glued a strip cut from two-cycle log paper. This strip is wide enough to carry two sets of graduations of the *A* scale. The upper divisions are lettered in black from 0.1 to 10 u./cc.; directly below, the same divisions are lettered in red from 1 to 100 u./cc. Below these two logarithmic scales there might be added a corresponding linear scale to be used in other types of calculations.

native scales may be secured on the vertical ruler (*O*) in such manner that any one can be selected by folding top ones back, like opening a book. A linear vertical scale may be used with equal success and would be in keeping with the recommendations of several authorities (5–7) who have shown that log dose plotted against diameter of inhibition zone will give a linear relationship for results obtained with the standard cup-plate assay. However, this instrument was developed for the new three-hour assay

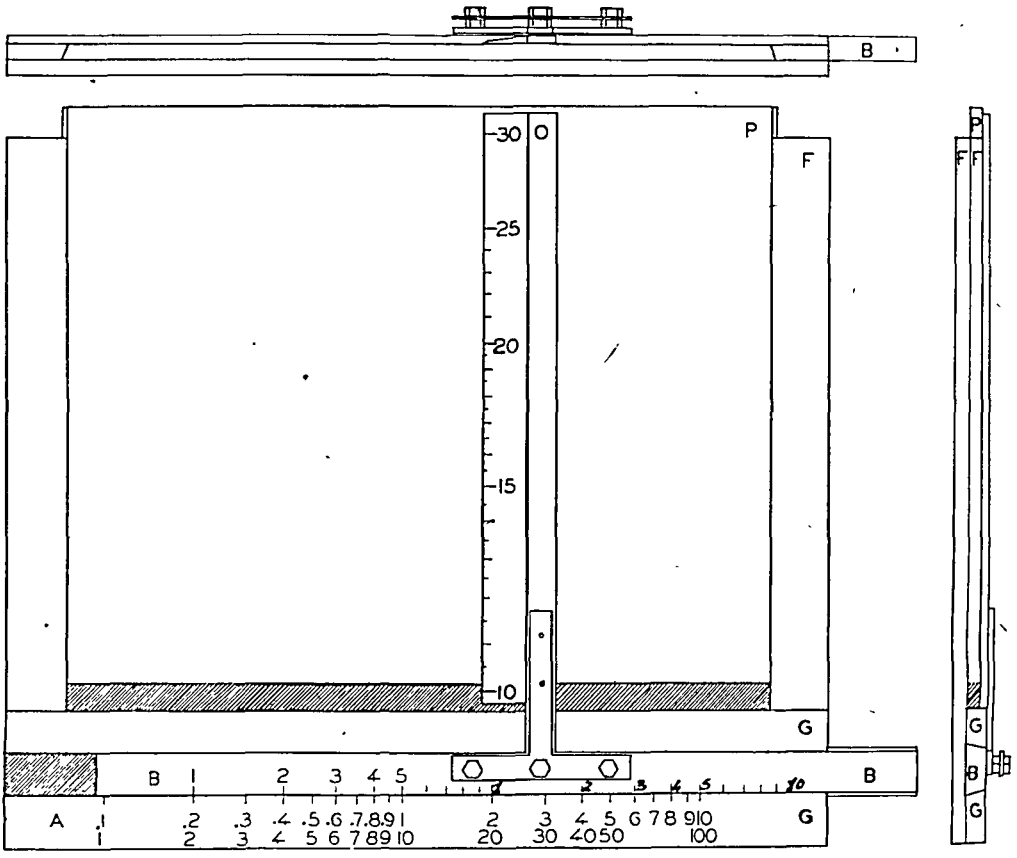

Fig. 1.—Detail drawing of graphical calculator.

A matching strip of the two-cycle log paper is glued to the movable ruler (*B*). The first cycle should be lettered from 1 to 10 in red and the second cycle from 1 to 10 in black so that the operator may match color between scales *A* and *B* and thus avoid confusion in placing the decimal point. A cursor may be fastened to the brass connector so that the reading line falls at the beginning of the second cycle on scale *B*.

To the vertical ruler is affixed a segment of a one-cycle log scale so chosen that the slope of the calibration curve plotted on board (*P*) shall fall at an angle of approximately 45°. When using cups 8 mm. in external diameter the diameters of zones of inhibitions range from 10 mm. to 30 mm.; therefore, for this type of cup-plate assay the usable part of the scale ranges from 10 to 30 at most. However, alter-

(8) and was found to work equally well with data obtained from the standard assay. The operation of the graphical calculator is not dependent upon strict linearity of the reference curve nor upon maintaining a log-log relationship between the vertical and horizontal scales.

The drawing board (*P*), to which unruled paper is fastened with adhesive cellulose tape, must be adjusted so that it can slide up and down over a sufficient range that, once a standard curve is established, it may be shifted to accommodate daily variations in the size of the standard zone.[2]

[2] The curve or line may also be shifted by slightly rotating the paper about any axis when it is desired to make use of the "two standard method." No mechanical aid has been provided in the present model to accomplish this, although a pin may be used to establish any desired axis of rotation.

The use of the calculator involves two steps: first, the plotting of a standard curve; second, reading concentrations of solutions under test from measured zone diameters. Once the reference curve is established it becomes an integral part of the calculator and may be used from day to day by sliding the panel *P* to the position indicated for the day by the size of the standard zone.

To Plot a Reference Curve.—Set the origin (1 at beginning of second cycle) of movable scale *B*, to face any known value of u./cc. on scale *A*. Record corresponding observed values of diameters of zones of inhibition on ordinate scale *O*, by marking a point on unruled paper attached to drawing board *P*.[3] Repeat for each known value. Two examples of the plotting of typical reference curves are given below.

Example 1.—Using data from Table I plot the reference curve.

TABLE I.—AVERAGE RESULTS FROM CUP-PLATE ASSAYS[a]

Known Concentrations of Penicillin, U./Cc.	Measured Zones Diameters, Mm.
0.25	14.80 ± 0.16
0.50	18.47 ± 0.14
0.75	20.43 ± 0.16
1.00	21.86 ± 0.13
1.50	23.59 ± 0.16
2.00	24.90 ± 0.16
3.00	26.50 ± 0.17
4.00	27.55 ± 0.19

[a] Routine measurements made at Cutter Laboratories by the standard Oxford cup-plate method according to the specifications of the Food and Drug Administration, using *Staph. aureus* F.D.A. strain No. 209P as the test organism. Each zone diameter represents an average taken from fifty daily standard curves.

[3] The frame of the calculator is assembled with machine screws and can be adjusted so that board (*P*) has no tendency to slip accidentally while plotting a curve.

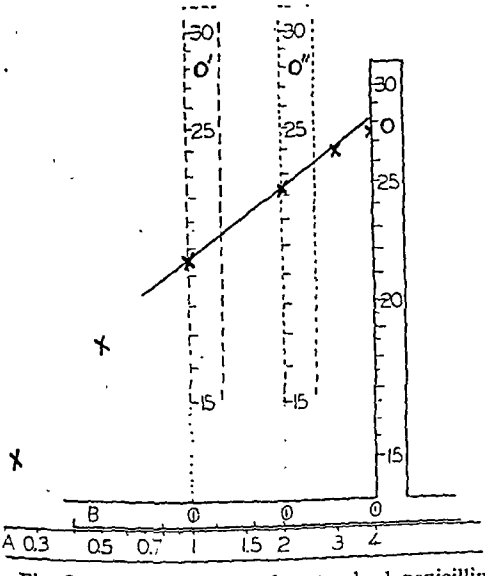

Fig. 2.—Reference curve for standard penicillin assay. The dotted scales O' and O" are set, respectively, for adjusting the instrument to a new standard and for reading an unknown concentration. (See Table I and Examples 1 and 3 in text.)

Secure paper to drawing board (*P*) which should be placed as shown in Fig. 1. Move the origin of scale *B* (beginning of second cycle of scale *B* shown in Fig. 1) successively to the values given in the first column of Table I. For each position a pencil mark is placed directly under the corresponding zone diameter, estimated on scale *O*.

Figure 2 shows this curve as plotted on paper attached to board *P*; the scale *O*, at the extreme right, shows the position of this scale and the attached scale *B* after plotting data of Table I.

Example 2.—Using the data from Table II plot the reference curve.

TABLE II.—AVERAGE RESULTS FROM 118 PHYSICALLY DEVELOPED PENICILLIN ASSAY PLATES

Penicillin, U./Cc.	Observed	Diameters of Zones of Inhibition Calculated[a]
0.50	13.28 ± 0.08	...
1.00	14.91 ± 0.08	14.93
2.00	16.23 ± 0.08	16.16
4.00	17.30 ± 0.08	17.45
8.00	18.93 ± 0.10	18.87

[a] Using first-degree orthogonal polynomials, as from Fisher and Yates, "Statistical Tables for Biological, Agricultural and Medical Research," through Anderson and Houseman (9).

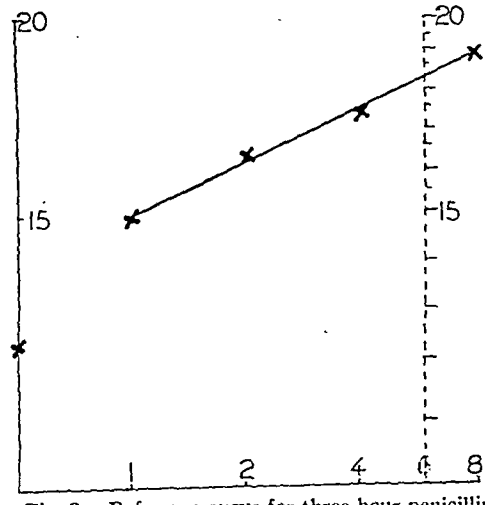

Fig. 3.—Reference curve for three-hour penicillin assay. The dotted scale is set for the reading of an unknown solution producing a zone 18.26 mm. in diameter. The concentration is seen to be 6 u./cc. Refer to Table II and Examples 2 and 4 of text.

This curve is plotted by following the procedure outlined in Example 2. *Figure 3 shows the completed curve.*

To Determine the Potency of Solution under Test.—(*a*) When the proper reference curve is on board *P*, move the scale *B* until the origin appears directly opposite the reading on the *A* scale equal to the value of the concentration of the standard solution of the day. (*b*) Slide the drawing board *P* up or down to set the reference curve directly under the value of the diameter of the standard

zone as read on the ordinate scale *O*. (*c*) Slide movable ruler *B* left or right until the reference curve appears directly under the division of the scale *O* corresponding to the measured diameter of the inhibition zone for the dilution under test. Then read u./cc. on scale *A* opposite the proper dilution factor on scale *B*. It will be noted that the use of dilution factors on scale *B* corresponds to simple multiplication with the *A* and *B* scales of ordinary slide rules.

Example 3.—Using the reference curve described in Example 1 and shown in Fig. 2 compute the concentration of penicillin corresponding to a zone size of 22.25 mm. when the average zone diameter for cups containing 1 u./cc. on the same set of plates is 19.6 mm.

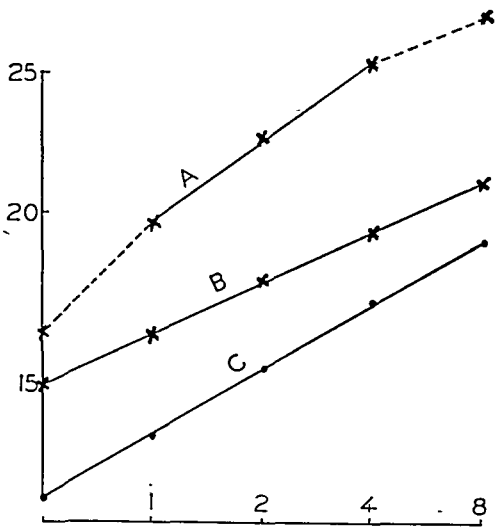

Fig. 4.—Reference curves for three different methods of cup-plate assay for penicillin. *A*, standard sixteen-hour method; *B* and *C*, physical development method (8); *B*, three hours' penicillin diffusion on plates incubated with *Staph. aureus;* *C*, two hours' penicillin diffusion on plates incubated with *B. subtilis*. The scales are the same as those shown in Fig. 1.

To make use of the unit zone given in Example 3 move scale *B* so that the origin corresponds with 1 u./cc. on scale *A*, then move board *P* so that the reference curve appears under 19.6 (position *O'*, Fig. 2). Without further movement of board *P* slide scale *B* until 22.25 as estimated on scale *O* coincides with the line; read the required concentration of 2 u./cc. opposite the origin of scale *B* (position *O"*, Fig. 2).

Example 4.—Using the reference curve mentioned in Example 2 and illustrated in Fig. 3 determine the concentration corresponding to a zone diameter of 18.26 mm. if a concentration of 1 u./cc. produces a zone of 14.9 mm. in diameter.

Figure 3 shows the calibration curve plotted as described above, the unit concentration is set at 14.93 which is practically equivalent to 14.9 so that it is not necessary to move board *P* to reset the standard, thus the concentration at 6.00 u./cc. is read directly by bringing scale *O* to the position shown in Fig. 3. If it is known, for example, that this solution was obtained by dilution, the value of the undiluted solution would be found opposite the known dilution factor.

ADVANTAGES OF THE GRAPHICAL CALCULATOR

The graphical calculator described above has been in regular use in our laboratory since July, 1946, and has proved satisfactory for different types of assays. It has been used effectively even when the function to be treated was not linear. By selecting the proper ordinate scale and by placing it in such a position that it is possible to cover any desired range, a variety of functions may be set up for the graphical computation of assays. Figure 4 shows three examples of different calibration curves which can be used on the same sheet of paper. This device also serves nicely as a means of plotting and recording results for comparison and reference.

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A Graphical Calculator for Statistical Analysis*

By J. DUFRENOY and FRANK M. GOYAN†

The graphical calculator originally developed for calculating penicillin concentrations from zone diameters in the three-hour cylinder-plate assay is shown to possess properties that recommend it for statistical analysis. By the introduction of two additional scales, the instrument is fitted to test data for deviation from normal distribution and to determine the mean and standard deviation from the mean of normally distributed values. Examples are included to demonstrate the advantages of graphical statistical analysis. A device for the semiautomatic plotting of data may be added if desired. The instrument is also useful in calculating values of LD_{50} from suitable data.

THE GRAPHICAL calculator previously described as expediting the plotting of reference curves for bioassays (1) can be adapted to the plotting of various functions by introducing the proper graduations on the horizontal and vertical scales. For the purpose of elementary statistical analysis, a probability scale, which may be made from a strip cut from a sheet of commercially available probability paper (2–4), is added as an additional horizontal scale, and a linear scale is provided for the vertical ruler. Thus equipped, the graphical calculator permits a direct visual test for deviations from normal distribution, together with direct reading of the mean and a rapid estimate of standard deviation from the mean of data that may be assumed to be normally distributed. The standard error of the mean or average ($S.E._{av.}$) is obtained from the standard deviation (σ) by the use of the equation,

$$(S.E._{av.}) = \sigma/\sqrt{n} \qquad (I)$$

where n represents the total number of quantities included in the average.

The theoretical justification and the significance of this method of calculating statistics are based upon well-established principles. The graphical calculator, modified as described above, exhibits the usual mathematical properties of arithmetic probability graph paper, which is commonly used in testing for normal distribution (3–5). Direct reading of mean values is possible because of the relationship between the mode of the normal distribution curve and the mid-point of the probability scale. Values of the mean *minus* the standard deviation, and values of the mean *plus* the standard deviation, are read directly opposite two fixed points on the probability scale. The position of these two points lying near the 16 per cent and the 84 per cent positions, respectively, is established from the fact that 68.27 per cent of normally distributed values fall symmetrically about the mean within the range established by values either greater or less than the mean by an amount equal to the standard deviation. Values determined in this way for the standard deviation are converted to the standard error of the average in the usual way; for example, as defined in the U. S. P. XIII monograph on Digitalis Tincture (6). It can be shown that this definition is equivalent to Eq. (I) by assuming that values of the standard deviation as read from the calculator are equivalent to values for the standard error, or the square root of the variance. The terms *normal distribution*, *standard error*, *standard deviation*, and *variance* are used in the sense established by modern treatises on statistical methods (7–9).

Although the graphical calculator offers no theoretical advantage over a sheet of probability paper, it provides a definite practical advantage in addition to the obvious one of greater convenience in plotting. This advantage arises from the fact that it is possible to designate fixed positions on the probability scale for various total numbers of values to be included in an average. These positions may be marked on the scale, or a mechanism consisting of a spring-activated plunger operating against a metal

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guide may be attached to the instrument in such a way that any selected series of positions on the probability scale is located automatically in the course of plotting the data. For relatively small populations, the only preliminary operation necessary to the operation of the instrument is to arrange the set of values to be studied in serial order, the smallest first, etc. Larger populations require preliminary grouping unless it is deemed safe to consider portions of the populations of values separately.

CONSTRUCTION OF THE GRAPHICAL CALCULATOR

The graphical calculator previously described for calculating the results of bioassays (1) is very easily modified for elementary statistical analysis. The essential new features shown in Fig. 1 are the horizontal probability scale (*A*), the vertical linear scale (*O*), and the cursor (*C*). The log scales described previously as occupying positions *A* and *B* are omitted from the drawing for the sake of clarity of presentation, although it is not desirable to remove these scales when modifying the instrument. Instead, the instrument is made to serve a double purpose by cementing a strip of probability paper below the log scales. The cursor (*C*) is provided for accurately reading the probability scale.

The linear vertical scale is made by fastening a strip of millimeter paper, or any other suitable linear scale, to the vertical ruler (*O*). It is necessary to provide for the temporary marking of the numerical values chosen for the graduations on this scale because each new problem may require a different range of values. The simplest method, of course, is to write numbers on the scale in pencil and to erase them when necessary. This scale should be constructed in a form that will permit it to be interchangeable with one or more log scales, which may prove useful for a wide variety of calculations.

Marking the Probability Scale.—In addition to the usual percentage values found on all probability scales, it is desirable to provide permanent or semi-permanent marks on the probability scale itself. These marks indicate the position of the cursor (*C*) when plotting data or reading the mean, the mean minus the standard deviation, or the mean plus the standard deviation. Such marks are made at the 50%, the 15.86%, and the 84.14% positions, respectively, to indicate the three reading positions.

The positions to be marked for plotting data may be calculated for each number of values likely to be encountered. Table I gives the location of these plotting points for odd total numbers of data from 3 to 25, inclusive. Marked points for even numbers of values are not required because no serious error is introduced by skipping the mid-point while plotting even numbers of data by making use of the positions established for the next higher odd number. Calculations of the entries in Table I are based upon the

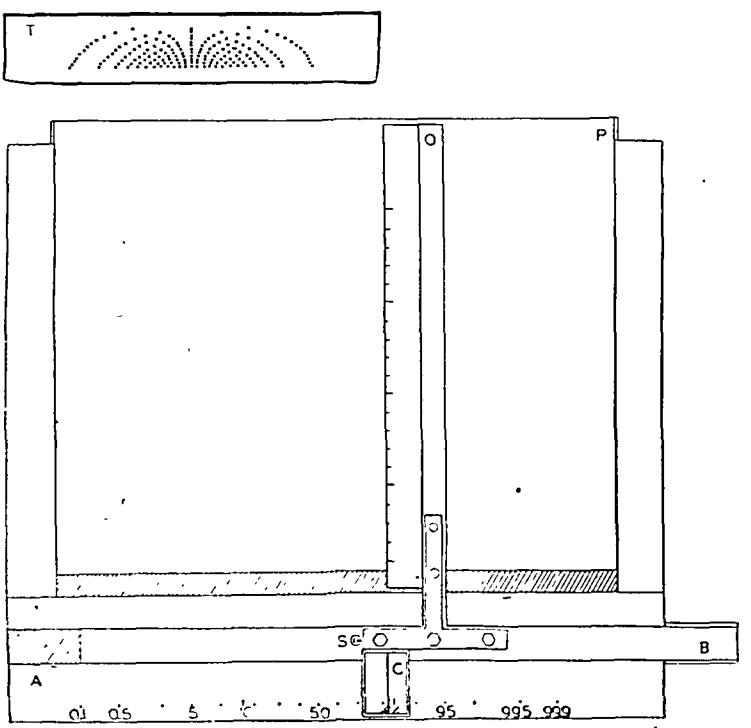

Fig. 1.—Top view of the graphical calculator adapted for the determination of the mean and standard deviation of a series of measurements assumed to be normally distributed. The insert T (not drawn to scale) shows the pattern of holes in a metal guide used to provide semi-automatic plotting.

idea of selecting the mid-point of the cumulative percentage range corresponding to each one of the population of values under consideration. For example, out of a population of five values, the first value represents 20%, and the mid-point of the range is 10%; the next value is thought to occupy the range from 20% to 40%, the mid-interval being the 30% position; the next position is 50%, etc. Twelve separate lines below the main probability scale would be required for marking the positions indicated by the columns of Table I, but if a color code is employed, two lines can be consolidated with some sacrifice of clarity. Each line must be labeled to show what points are to be placed under the reading line of cursor *C* when plotting a set of data containing the number of values indicated by the label.

length of steel rod chosen so that it slides freely up and down through a piece of brass tubing which is forced through a perpendicular hole in the moving part (*B*). A slot is cut under the path of the plunger through the base of the calculator; horizontal movement of the sliding ruler (*B*) causes the rounded tip of the plunger to slide from one hole in the guide to the next, coming to rest in each successive hole of whatever horizontal line of the guide was selected. The tension of the coil-spring holding the plunger against the guide is so adjusted that the sliding ruler (*B*) comes to a stop each time the rounded end of the plunger comes to rest in one of the holes in the metal guide; slight additional pressure, tending to slide the ruler (*B*), causes the plunger to progress horizontally to the next position.

TABLE I.—LOCATION OF SUCCESSIVE POSITIONS ON THE PROBABILITY SCALE FOR PLOTTING ODD NUMBERS OF DATA IN GROUPS OF 3 TO 25

Serial No.	Number of Data											
	3	5	7	9	11	13	15	17	19	21	23	25
1	16.7	10.0	7.1	5.6	4.5	3.8	3.3	2.9	2.6	2.4	2.2	2.0
2	50.0	30.0	21.4	16.7	13.6	11.5	10.0	8.8	7.9	7.1	6.5	6.0
3	83.3	50.0	35.7	27.8	22.7	19.2	16.7	14.7	13.1	11.9	10.9	10.0
4	...	70.0	50.0	38.9	31.8	26.9	23.3	20.6	18.4	16.7	15.2	14.0
5	...	90.0	64.3	50.0	40.9	34.6	30.0	26.5	23.7	21.4	19.6	18.0
6	...	...	78.6	61.1	50.0	42.3	36.7	32.3	28.9	26.2	23.9	22.0
7	...	...	92.9	72.2	59.1	50.0	43.3	38.2	34.2	31.0	28.3	26.0
8	...	...	...	83.3	68.2	57.7	50.0	44.1	39.5	35.7	32.6	30.0
9	...	...	...	94.4	77.3	65.4	56.7	50.0	44.8	40.5	37.0	34.0
10	...	...	...	...	86.4	73.1	63.3	55.9	50.0	45.2	41.3	38.0
11	...	...	...	...	95.5	80.8	70.0	61.8	55.2	50.0	45.6	42.0
12	...	...	...	...	...	88.5	76.7	67.7	60.5	54.8	50.0	46.0
13	...	...	...	...	...	96.2	83.3	73.5	65.8	59.5	54.4	50.0
14	...	...	...	...	...	...	90.0	79.4	71.1	64.3	58.7	54.0
15	...	...	...	...	...	...	96.7	85.3	76.3	69.0	63.0	58.0
16	...	...	...	...	...	...	...	91.2	81.6	73.8	67.4	62.0
17	...	...	...	...	...	...	...	97.1	86.9	78.6	71.7	66.0
18	...	...	...	...	...	...	...	...	92.1	83.3	76.1	70.0
19	...	...	...	...	...	...	...	...	97.4	88.1	80.4	74.0
20	...	...	...	...	...	...	...	...	...	92.8	84.8	78.0
21	...	...	...	...	...	...	...	...	...	97.6	89.1	82.0
22	...	...	...	...	...	...	...	...	...	...	93.5	86.0
23	...	...	...	...	...	...	...	...	...	...	97.8	90.0
24	...	...	...	...	...	...	...	...	...	...	...	94.0
25	...	...	...	...	...	...	...	...	...	...	...	98.0

Mechanism for Automatic Plotting.—Table I was used in preparing the metal guide shown in the insert (*T*) of Fig. 1. The pattern of dots represents holes drilled in a piece of sheet brass at positions determined by plotting entries in Table I on probability paper cemented to the brass; each column was plotted on a separate horizontal line. By means of springs, the guide is held in contact with the under surface of the calculator directly under the plunger (*S*) in such a way that its longer dimension is parallel to the probability scale. It may be moved forward or backward in a direction perpendicular to the scale. An extension of this metal guide (not shown) is provided with holes that engage pins secured to the base of the calculator. This precaution eliminates the possibility of a shift in the position of the guide once it has been properly placed as determined by an auxiliary scale attached to the extension.

The plunger (*S*) is constructed from a short

USE OF THE GRAPHICAL CALCULATOR

Treatment of Data Without the Use of Table I.—The first step in using the graphical calculator is to arrange all of the values to be studied as a single group, in serial order of increasing or decreasing magnitude. It is a well-known property of the normal distribution that, out of one hundred values serially arranged, the first and last sixteen will normally have values that deviate from the mean by amounts equal to or greater than the standard deviation. As an example, measured diameters of one-hundred inhibition zones produced by the action of penicillin solutions of unit concentration in the standard cylinder-plate assay are serially arranged in Table II. The fiftieth value is 21.50 which corresponds to the value of the arithmetic mean (21.51). The sixteenth value is 20.75 which agrees with the value 20.76 calculated by subtracting the value of the

standard error (computed by conventional methods) from the mean. The eighty-fifth value is 22.25 which is in agreement with the value calculated by adding the standard error to the mean.

The selection of one hundred measurements was made to illustrate some of the properties of the probability scale. For one hundred measurements, each measurement accounts for 1% on the probability scale, although the actual point is placed at mid-intervals. For example, the first point is plotted at a position corresponding to the 0.5% division on the probability scale. The second point is plotted to correspond to the 1.5% position; the fiftieth point, to the 49.5% position; etc. This principle may be applied to any number of points after computing, from the serial number of each point, the corresponding percentage value. The method of making this calculation is discussed in connection with Table I.

TABLE II.—DIAMETER (x) OF 100 INHIBITION ZONES PRODUCED BY PENICILLIN SOLUTIONS OF UNIT CONCENTRATION IN THE STANDARD CYLINDER-PLATE ASSAY[a]

x Mm.	x Mm.	x Mm.	x Mm.
19.70	21.10	21.50	21.90
19.80	21.15	21.50	21.95
19.90	21.15	21.55	21.95
20.10	21.15	21.55	21.95
20.20	21.20	21.55	22.00
20.30	21.20	21.55	22.00
20.40	21.25	21.60	22.00
20.50	21.25	21.60	22.10
20.55	21.30	21.60	22.20[d]
20.55	21.30	21.65	22.25
20.60	21.30	21.65	22.25
20.65	21.35	21.70	22.30
20.65	21.35	21.70	22.35
20.70	21.35	21.70	22.40
20.75	21.35	21.75	22.45
20.75[b]	21.40	21.75	22.50
20.80	21.40	21.75	22.55
20.85	21.45	21.80	22.55
20.85	21.45	21.80	22.70
20.85	21.45	21.80	22.80
20.85	21.45	21.85	22.90
20.90	21.50	21.85	22.95
20.95	21.50	21.85	23.20
21.10	21.50	21.90	23.30
21.10	21.50[c]	21.90	23.40

[a] Random selection from routine assays serially arranged. Data from 4 consecutive months, Cutter Laboratories.
[b] 16th value. [c] 50th value. [d] 84th value

The first step in plotting the points corresponding to the zone diameters listed in Table II is to mark the linear vertical scale (O) in the manner indicated by the ordinate scale shown in Fig. 2, and to secure a piece of paper to the drawing board of the instrument. The vertical scale moves with the cursor (C) which is placed so its reading line coincides with the 0.5% position on the probability scale; the first point is plotted opposite a division of the vertical scale corresponding to a zone diameter of 19.70 mm. Each successive point is plotted from Table II in the same manner at intervals of 1% on the probability scale;

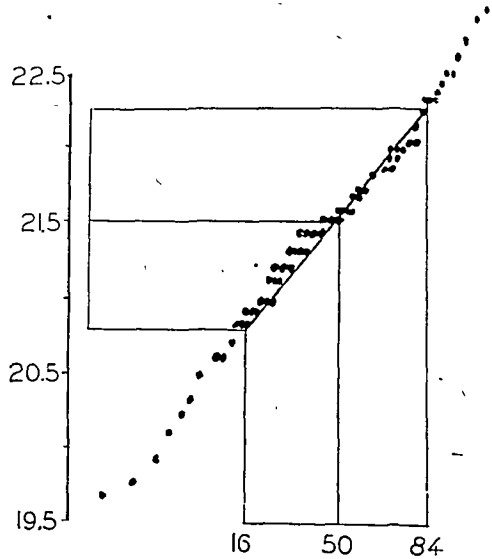

Fig. 2.—Graphical determination of the mean and standard deviation of the diameter of 100 inhibition zones from the standard cylinder-plate assay of penicillin solutions containing 1 unit/cc. The abscissa represents the probability scale; the ordinate, zone diameters (mm.).

the second point, at 1.5%; the third, at 2.5%; the next, at 3.5%; etc., up to 99.5% on the probability scale.

The best fitting straight line is drawn through the points as shown in Fig. 2. The cursor is set opposite the 50% division on the probability scale for the purpose of reading the mean value at the intersection of this line with the vertical scale. From Fig. 2 it may be observed that the value of the mean is read at 21.5 mm. The cursor is then set above the reading mark near the 16% graduation of the probability scale, and the corresponding value of the mean minus the standard deviation is read on the linear scale as 20.8 mm. Therefore, the value of the standard deviation from the mean is 21.5 − 20.8, or 0.7 mm. A similar reading is made when the cursor coincides with the reading mark near the 84% division of the probability scale, and the value of 22.2 mm. is noted. This checks the previous reading, and it will be observed that the standard deviation from the mean is one-half of the difference between the 84% reading (22.2) and the 16% reading (20.8).

Treatment of Data with the Aids Derived from Table I.—The use of Table I eliminates the need for calculating positions on the probability scale for each successive value in a series. For odd numbers of data less than 26, percentages found in the table may be used directly, and for even numbers the percentages chosen from the column referring to the next highest number are used with the omission of the mid-point of scale (50% division) in plotting. For numbers of values greater than 25, it has been found convenient to group the data so as to supply 25 or fewer groups. Proper grouping is necessary in

order to facilitate plotting with the aid of Table I or the automatic guide described above, and grouping errors can be minimized by selecting as large a number of groups as practicable.

In every case it is necessary to arrange the data in serial order of increasing or decreasing magnitude before grouping. Serial arrangement may be conveniently accomplished by tabulating the data in vertical columns covering the range of the values. Only the last figure of each value need be tabulated in its proper column at an appropriate relative position that provides space to arrange all values that may accumulate in the column.

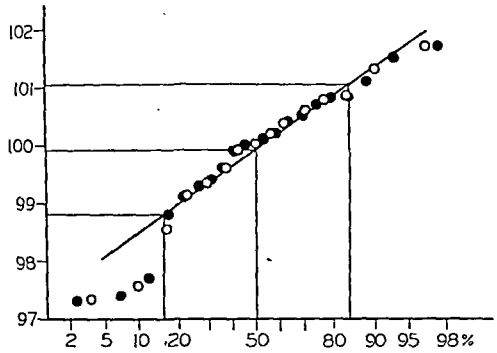

Fig. 3.—Graphical determination of the mean and standard deviation of the iodometric determinations of penicillin reported by Mundell, *et al.* (10). Open circles represent averages of 15 groups of 4 values each; dark circles represent every third value, beginning with the second, taken from the same data after serial arrangement. Per cent recovery of penicillin is plotted against reading points automatically selected by the instrument.

For the purpose of illustration, the sixty comparative assays of crystalline penicillin G by iodometric titration given by Mundell, Fischbach, and Eble (10) were selected for study. Two general methods of grouping are illustrated in Fig. 3, where the open circles represent 15 points plotted from the averages of groups of four values selected after serial arrangement. The black circles represent 20 values selected by taking every third value beginning with the second in the serially arranged list of values. It will be noted that both methods are in agreement, and that, for the most part, the data are normally distributed. The few points that fail to fall on the straight line are given little weight in fitting the line from which the mean is read as 99.93, a value closer to the theoretical 100% recovery than the value of 99.82 obtained by Mundell, *et al.*, by conventional methods. A difference between the arithmetic mean and the value of the mean obtained from the best visual fit to the straight line indicating normal distribution is to be expected if some of the values are given little weight because they do not fall on the normal distribution curve. For the same reason, values for the standard deviation are slightly smaller than those calculated in the usual way (in this case, 1.11 instead of 1.20).

Use of a Vertical Logarithmic Scale.—The calculator is so constructed that a logarithmic scale may be used interchangeably with the linear vertical scale for the purpose of studying data that are best handled by means of logarithmic probability paper (3, 5), such as the plotting of log dose of a poison against the percentage of test organisms succumbing (11, 12). A straight line, visually fitted to such a plot, permits a direct estimate of LD_{50} from the point where the line intersects the vertical log scale when the reading line of the cursor coincides with the mid-point of the probability scale. LD_{95} may also be read by setting the cursor over the 95% position on the probability scale. While this method is not as highly refined as methods developed for the use of the probit scale (8, 13), it has the advantage of simplicity and speed. A probit scale may be drawn for use as an auxiliary probability scale if desired. To construct such a scale, the reading point near the 16% position on the probability scale, described above, is probit 4; the 50% division is probit 5, and the remaining reading point (84.14%) is probit 6. Equal subdivisions and linear extensions complete the probit scale.

SUMMARY AND CONCLUSIONS

The graphical calculator provides a practical way of making use of the known mathematical properties of arithmetic probability paper in testing for deviations from normal distribution and in estimating the mean and the standard deviation from the mean. Equipped with a vertical log scale, the instrument may be used to advantage in estimating other statistics (i.e., LD_{50}). In general, the graphical calculator may be thought of as providing a rapid visual estimate of quantities that may later require more refined analysis. In testing for significance, for example, it is important to use the values obtained from the graphical calculator with the same degree of caution that would be applied to the use of the same values calculated in any other way. However, there can be no doubt that the data plotted in Fig. 3 are much more illuminating than pure analytical calculations because of the clear demonstration of their relation to normal distribution.

This example illustrates the importance of the geometrical vs. the arithmetical approach in statistical analysis, emphasised by Treloar (14). by the inclusion of the following

quotation from Francis Galton: "It is always well to retain a clear geometric view of the facts when we are dealing with statistical problems, which abound in pitfalls, easily overlooked by the unwary, while they are cantering gaily along upon their arithmetic." Rissik (3) makes the same point more directly in the statement: "The advantages attaching to the use of probability graph paper in the solution of statistical problems involving a determination of the mean and standard deviation of a series of quantitative data are so great that, in the author's opinion at any rate, the graphical method is nearly always to be preferred."

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The Solubilization of Quinine by Bile Salts*

By S. MUKHERJEE and R. P. BANERJEE†

From turbidimetric titrations of bile salts with quinine and turbidity-concentration curves of colloidal solutions of quinine-dehydrocholic acid and tauroglycocholic acid complexes, it has been concluded that solubilization takes place through the formation of micelles composed of quinine and bile salts. Two types of micelles are envisaged; primary micelles built up by the association of quinine with bile salt molecules or with very small micelles; and secondary micelles formed of quinine molecules and large micelles of bile salts. The primary micelles are easily diffusible while secondary micelles form irreversible precipitates. Solubilization is accompanied with the formation of primary micelles. This would explain the difference in solubilizing powers of sodium dehydrocholate and tauroglycocholate.

IT HAS LONG been known that bile salts can render water-soluble many water-insoluble substances, e.g., fats,, fatty acids, alcohols, ethers, etc. The mechanism by which this solubilization takes place has evoked much interest. The earliest hypothesis for explaining this phenomenon is the "choleic acid principle" of Wieland and Sorge (1), which postulates the formation of soluble co-ordination complexes of these substances with the bile salts. It appears from the recent work of McBain and co-workers (2, 3) that solubilization can also be brought about by many other detergent substances which can be classed as colloidal electrolytes. According to the hypothesis put forward by these authors, solubilization is effected by the sorption of the insoluble substance on the micelles of the detergent solution. Thus, the greater the micelle-forming ability of a detergent, the greater would be its solubilizing power. Roepke and Mason (4) have recently shown that, above a certain "critical" concentration, solutions of bile salts also contain micelles, and these can therefore be regarded as colloidal electrolytes.

Mukherjee, Banerjee, and Basu (5) have shown that bile salts can solubilize quinine under favorable conditions. When a solution of quinine salt is added gradually to an alkaline bile salt solution, a stable colloidal solution is formed, so long as the mixture remains alkaline and the molar ratio of qui-

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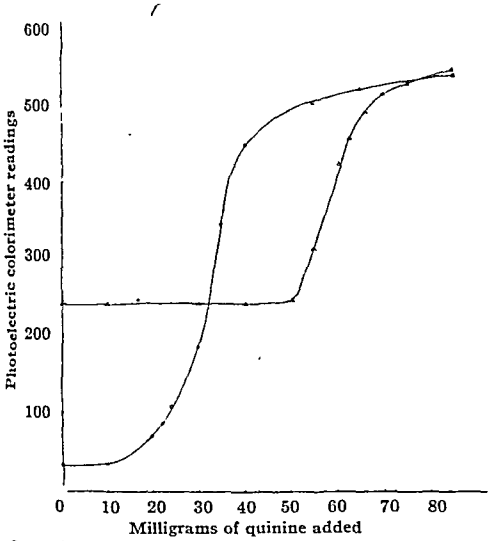
† The authors wish to thank Dr. U. P. Basu for his interest in this work.

nine to the bile acid does not exceed about 1:4. When such a colloidal solution is dialyzed in a bag of cellophane, swollen in 64 per cent zinc chloride solution, both the quinine and the bile acid can diffuse out into the dialysate. An investigation of the nature of this colloid and the mechanism of the solubilization appeared to be of interest.

EXPERIMENTAL

Turbidimetric titrations of sodium dehydrocholate and tauroglycocholate with quinine were carried out in the following manner: To 25 ml. of a 0.0468 *M* solution of either the dehydrocholate or the tauroglycocholate was added gradually a 0.068 *M* solution of quinine hydrochloride, and the change in the turbidity of the resulting mixture was followed using a Klett–Summerson photoelectric colorimeter. The titration curves are shown in Fig. 1.

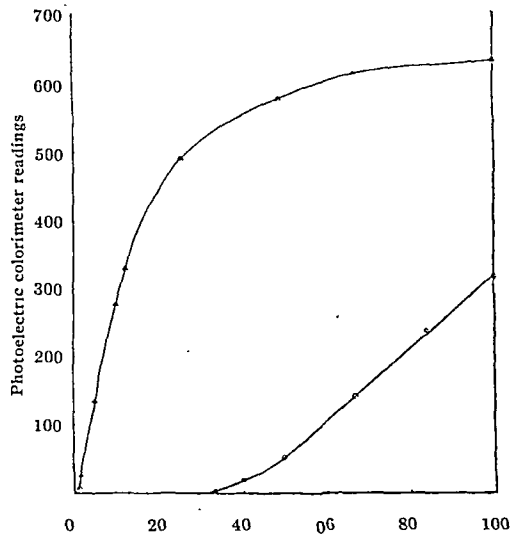


⊙ = Sodium dehydrocholate. △ = Sodium tauroglycocholate

Fig. 1.—Turbidimetric titrations with quinine hydrochloride.

A study was also made of the changes of turbidity of these colloids with dilution. The respective colloidal solutions were prepared by gradually adding 8.5 ml. of a 0.068 *M* solution of quinine hydrochloride to 50 ml. of a 0.0468 *M* solution of sodium dehydrocholate or tauroglycocholate. The colloidal solutions thus obtained were diluted with water to different extents, and the diluted solutions were set aside for two hours, with frequent stirring, before taking readings in the photoelectric colorimeter. The turbidity-concentration curves have been shown in Fig. 2, the concentrations being expressed as percentages of the undiluted colloids.

The initial lag in the quinine-dehydrocholate titration curve (Fig. 1) indicates that micelle formation, leading to turbidity, takes place only after a limiting concentration of the complex is reached. The number of micelles increases with further increase in the concentration of quinine. Again, dilution of quinine-dehydrocholate colloid (Fig. 2) is attended by a



⊙ = Quinine + Sodium dehydrocholate colloid
△ = Quinine + Sodium tauroglycocholate colloid

Fig. 2.—Turbidity-concentration curves of quinine + bile salt colloids.

regular, almost linear, fall in turbidity, the solution becoming practically clear and free from micelles when the concentration is about 30% of the original. These results indicate that the micelles of the quinine-dehydrocholate complex, obtained as above, possess a reversible-equilibrium solubility, as would be expected of a true colloidal electrolyte.

The observations of Mukherjee, Banerjee, and Basu (5) that, during dialysis of the colloids, quinine and bile salt diffuse out through the membrane simultaneously, and that the first day's dialysate also becomes turbid, give support to the view that the solubilization of quinine is effected by the formation of micelles (containing both quinine and the bile acid) which can penetrate the membrane.

McVain, Merrill, and Vinograd (2), working with the water-insoluble dye, Yellow AB (phenylazo-2-naphthylamine), found that sodium dehydrocholate had no solubilizing action on this dye, while sodium cholate, taurocholate, and deoxycholate possessed marked solubilizing power. They explained the difference on the basis of differences in the micelle-forming abilities of these bile salts.

Work in this laboratory (5) has shown, however, that both sodium dehydrocholate and tauroglycocholate can solubilize quinine, and that the dehydro-

dressing, especially when used on exposed areas, as for impetigo.

Reapplication of Furacin Soluble Dressing may be made as often as four times daily for such conditions as impetigo, to once or twice daily, or even less often, for wounds and burns.

SUMMARY

The antibacterial power of compounds containing the furan nucleus is markedly increased by addition of a 5-nitro group.

Nitrofurazone (Furacin) is the first nitrofuran to be made available for clinical use. It is either bacteriostatic or bactericidal to a wide range of organisms, including both Gram-negative and Gram-positive bacteria, both *in vitro* and *in vivo*. It has low systemic and local toxicity. Sensitization may be encountered.

Furacin is now available as Furacin Soluble Dressing for the topical treatment of wound and surface infections.

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Book Review

Bacteriology—Laboratory Directions for Pharmacy Students, Second Edition. Edited by MILAN NOVAK and ESTHER MEYER. C. V. Mosby Company, St. Louis, Mo., 1947. 248 pp. 26.5 x 19 cm. Price $2.75.

This outline was designed to present a systematic introduction of laboratory procedures to be used by beginning students in the study of bacteriology. Since time is an important factor in dealing with living things, it is imperative that the schedule used in the daily study of microorganisms be carefully planned in advance. This is particularly true when the work of two or more days must overlap due to the slow rate of multiplication of many bacterial and mold species.

This book presents detailed instructions of techniques to be followed throughout an entire laboratory course in bacteriology. In all, thirty-six sections, or periods, covering the various phases of handling bacteria, their significance in infections, differential diagnostic methods for identifying bacteria by stains, media, serology, etc., are presented in exacting details. Consideration is also given to the more recent advancements made in bacteriology to include the effects of sulfa drugs, antibiotics, and antiseptics on microorganisms. Details and interpretations of test procedures involving sterility testing of pharmaceutical preparations, fungicidal and fungistatic tests on ointments, powders and liquids as well as microbiology of food and pharmaceutical syrups are timely subjects which should prove to be of valuable interest to pharmacy students.

While the outline may be considered a complete presentation of laboratory methods, the reviewer questions the desirability of including discussions of various phases of bacteriology in reference to theories, historical development, definitions, etc. These points are often interspaced in the daily outline of laboratory directions, thereby requiring time to read and pick out the essentials assigned for the day's program. Since reference is made in the introductory chapter to the effect that the laboratory work is to be regarded as a supplement to regular didactic lectures and frequent demonstrations, it would appear more desirable to discuss the general principles of bacteriology at these meetings.

It is also anticipated that each student will personally have the opportunity to prepare at least one bacteriological medium throughout. In addition, the principles of sterilizing by autoclaving, dry heat, or filtration can only be impressed upon the student by his own participation in these procedures.

In general, the outline is a complete one and should prove to be a valuable aid to the teaching of laboratory bacteriology, not only to pharmacy students, but to medical students as well. A novel feature of the manual is the modern loose-leaf plastic binding which permits removal or reinsertion of pages when necessary.—C. A. LAWRENCE.

Scientific Edition

JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION

JUSTIN L. POWERS, EDITOR, WASHINGTON, D. C.

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Thyroid Function and the Duration of Sodium Pentobarbital Anesthesia*

By H. E. EDERSTROM

Thyroidectomy in adult female rats lengthened the duration of sodium pentobarbital anesthesia. Thyroid administration to normal rats did not change significantly the length of anesthesia. Thiouracil feeding failed to change the length of the anesthetic period. In dogs, thyroidectomy had no significant effect on the duration of pentobarbital anesthesia. Thyroid treatment of thyroidectomized dogs did not alter the length of anesthesia.

SEVERAL AGENTS have been described as antagonistic to barbiturate anesthesia, among them being drugs of the sympathetic-mimetic type. Westfall (1) reported the antagonistic effect of epinephrine on sodium pentobarbital anesthesia, and Freireich and Landsberg (2) recommended amphetamine for barbiturate poisoning. On the other hand, depressing agents, such as cold sufficient to lower body temperature, have been found to prolong the effects of barbiturate action by Raventos (3), Gaylord and Hodge (4), and Fuhrman (5).

These findings suggest that the general state of body metabolism may be a factor in determining the length of the sleep produced by this group of drugs. Rowbottom (6) implied that thyroxine could be used to counteract barbiturate toxicity, but Scarborough (7) reported that no significant change in the sleeping time of rats given pentobarbital could be produced by feeding the animals 0.03 Gm. desiccated thyroid daily. In view of these conflicting statements, it was considered that the role of thyroid in barbiturate anesthesia should be investigated further.

EXPERIMENTAL

Rats.—The methods used to alter thyroid function in rats were thyroidectomy, thyroid feeding, and thiouracil feeding. A total of 38 female and 16 male rats four months of age or older were thyroidectomized. Fourteen days or more postoperatively these were given intraperitoneal injections of 25 mg./Kg. sodium pentobarbital at intervals of one week or longer. Controls of comparable age and sex were injected with the same dose. Termination of

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the sleeping period was recorded when the rat voluntarily righted itself from the inverted position.

Thyroid-fed rats were given daily about 50 mg. desiccated thyroid (Parke–Davis, 50%. stronger than U. S. P.) in a sugar solution administered orally. This dose of thyroid was reported by Warkentin, *et al.* (8) to increase the B. M. R. of rats 30% to 50%. After sixteen days of this regimen injections of pentobarbital were begun, and thyroid feeding continued throughout the experiment. Thiouracil-fed rats were given 0.1% of the drug in the diet for twenty-eight days prior to, and throughout the period of barbiturate injections. This concentration of thiouracil given in drinking water was found to depress the B. M. R. of rats 23.7% in fourteen days by Reineke, Mixner, and Turner (9).

Dogs.—A second series of experiments concerned the influence of thyroid function on the length of pentobarbital-induced sleep in dogs. One male and 5 female adult mongrels weighing from 5.3 to 11.7 Kg. were used. The dogs were kept indoors and fed a diet of commercial dog food throughout the course of the investigation. During the anesthetic period the animals were kept at room temperatures varying from 22° to 27°. The termination of sleep was more difficult to establish in the dog than in the rat because of the long semiconscious state persisting after the deep anesthetic effect had worn off. Consequently, the end of the sleeping period was set arbitrarily at the time when the dog had regained sufficient coordination to walk on a polished floor.

One male and three female dogs were thyroidectomized, and permitted about four weeks' postoperative recovery. Intraperitoneal injections of 32.5 mg./Kg. sodium pentobarbital were then given weekly for seventeen consecutive weeks. The drug was then discontinued for several weeks, following which 65 mg. desiccated thyroid per day was administered orally. After one week of thyroid treatment, injections of pentobarbital were resumed for ten consecutive weeks, during which thyroid administration was continued.

Two normal female dogs were given 17 consecutive injections of pentobarbital at weekly intervals as described above. These animals were then thyroidectomized and permitted to recover about four weeks postoperatively. Pentobarbital injections were then given weekly for ten consecutive times.

RESULTS

Rats.—Table I summarizes the data obtained from the experimental and control rats. The thyroidectomized females show the greatest change, the average sleeping time in these being prolonged over that of the controls by twenty-one minutes. These data were treated statistically and the standard error of the difference between the two means determined by methods described by Davenport and Ekas (10). The difference between the means exceeded twice the standard error of the difference by about 17%, suggesting that a true difference existed in the duration of anesthesia between experi-

TABLE I.—DURATION OF PENTOBARBITAL ANESTHESIA IN RATS

Condition of Animals	Total No. of Rats		Total No. of Trials		Average Sleeping Time		Mean Difference		Standard Error of Difference	
	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
Thyroidectomized	16	38	42	105	122	167	18	21	10.82	8.55
Controls	14	25	30	59	104	146	..	..	...	...
Thyroid fed	0	13	0	29	0	121	..	10.1	...	12.58
Controls	0	9	0	23	0	131	..	..	...	...
Thiouracil fed	0	29	0	83	0	154	..	14.0	...	9.67
Controls	0	9	0	29	0	140	..	..	...	...

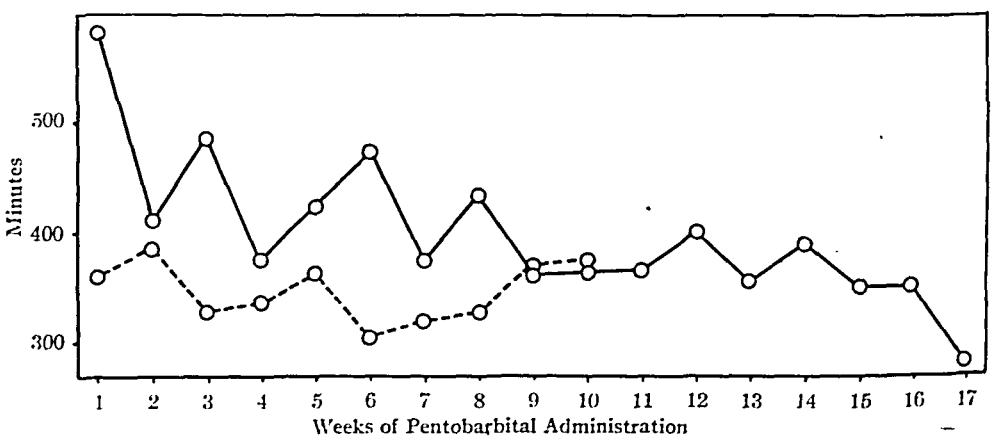

Fig. 1.—Solid line shows average sleeping time of 4 dogs after thyroidectomy. Dotted line shows sleeping time of same dogs during administration of 65 mg. thyroid per day.

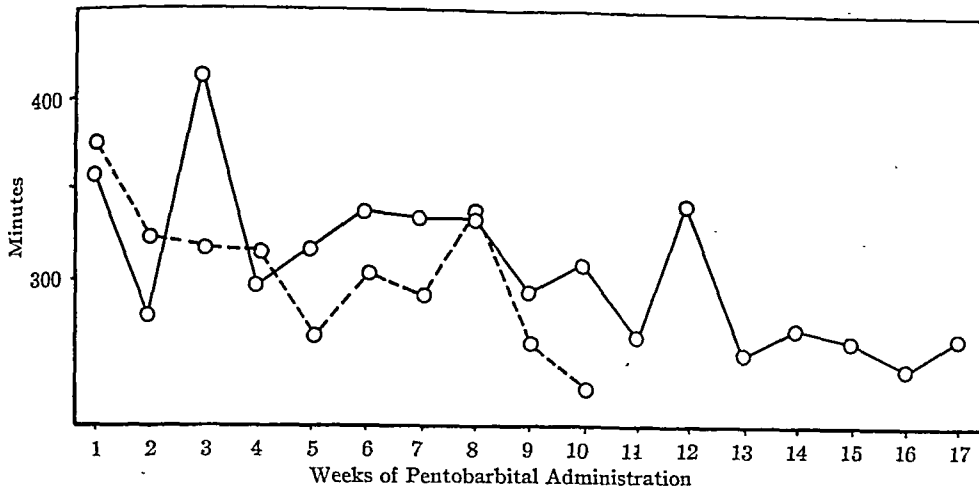

Fig. 2.—Solid line shows average sleeping time of 2 dogs before thyroidectomy. Dotted line shows sleeping time of same dogs postoperatively.

mentals and controls. The operated males likewise slept slightly longer than controls, but application of the statistical procedure above disclosed that no significant difference existed between means of the operated and control rats. Thyroid-fed females did not sleep quite as long as the controls, but here again statistical treatment failed to disclose a significant difference. The mean of thiouracil-fed females suggested that these slept longer than controls, but statistically this could not be established as a true difference (Table I).

Dogs.—The graph in Fig. 1 compares the sleeping time of four thyroidectomized dogs with that found in the same dogs during thyroid administration. Although a decrease in sleeping time appears to accompany thyroid feeding, this difference can be ascribed to barbiturate tolerance developed in the course of the experiment. This is indicated by the fact that the graph of sleeping time during thyroid administration continues at about the same level as the last few trials previous to hormone feeding. Ettinger (11) reported a similar tolerance to pentobarbital in dogs.

The graph in Fig. 2 shows the average duration of pentobarbital anesthesia in two normal dogs compared with that found in the same animals after thyroidectomy. No significant difference is indicated by these data.

DISCUSSION

Since the thyroid gland controls metabolism to a considerable degree, changes in its function could be expected to influence organs involved in the detoxification and excretion of pentobarbital. However, only in thyroidectomized female rats could a significant difference in the length of action of pentobarbital be demonstrated. Thyroidectomy, thiouracil feeding, and thyroid administration did not produce marked changes in the duration of pentobarbital anesthesia in the other experiments.

SUMMARY

1. Thyroidectomy in female rats tended to prolong the sleeping period resulting from sodium pentobarbital administration.

2. Thyroidectomy in male rats did not significantly alter the length of the sleeping time.

3. Administration of thyroid to rats did not significantly alter the period of sleep induced by pentobarbital.

4. Thiouracil feeding did not influence sleeping time to any significant degree.

5. In dogs, neither thyroidectomy nor thyroid administration to the operated dogs had any appreciable influence on the length of sleep induced by sodium pentobarbital.

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The Preparation of 1-Diethylamino-2-(2,4,6-Triiodophenoxy)ethane Hydrochloride*

By ALFRED BURGER

THE DRUG 1-diethylamino-2-(2,4,6-triiodophenoxy)ethane hydrochloride, first synthesized in our laboratory (1), has been the subject of a number of metabolic, toxicity, and chemotherapeutic studies (2) under the code name of B-9. It showed promising activity in experimental tuberculosis (2) and had the most favorable pharmacological properties in an extended series of compounds of related structure (3).

On several occasions the author has had to prepare considerable batches of the drug to supply physicians for preliminary clinical trials. Since a number of agencies have recently expressed interest in testing the drug it appears advisable to compile revised directions for its preparation, incorporating the experiences obtained in larger runs.

The success of the Williamson type condensation described below depends largely on the use of pure starting materials and the avoidance of overheating in the various phases of the reaction.

EXPERIMENTAL

β-Diethylaminoethyl Chloride Hydrochloride.—In a 2-L. three-necked flask equipped with a sealed mechanical stirrer, dropping funnel, and reflux condenser connected to a gas trap place a mixture of 200 Gm. of β-diethylaminoethanol and 300 cc. of dry benzene. Cool in a freezing mixture, and drop a solution of 262 Gm. of thionyl chloride in 200 cc. of dry benzene into the stirred mixture over a period of three to four hours. Remove the ice bath, place the flask on a steam bath, and heat the mixture to reflux for four hours, maintaining stirring as long as the accumulating thick mass of crystals will permit.

Cool the mixture, filter the hydrochloride with suction, wash it several times with cold acetone, and finally with a mixture of one part acetone and one part ether. Dry well in a vacuum desiccator and recrystallize the salt from about 400 cc. of ethanol. Continue recrystallizing until the melting point of the now nonhygroscopic salt reaches 207–208°. The yield of purest salt is 100 Gm. or better.

Additional fractions of lesser purity may be obtained by concentration of the mother liquors and used for other purposes. Only the best material should be used in the next step.

1 - Diethylamino - 2 - (2,4,6 - Triiodophenoxy)-ethane Hydrochloride (B-9).—Prepare a solution of 19.0 Gm. of clean sodium in 450 cc. of 99% methanol, and dissolve in it 190 Gm. of pure 2,4,6-triiodophenol. As soon as this compound has dissolved, add a solution of 70 Gm. of β-diethylaminoethyl chloride hydrochloride in 150–200 cc. of methanol with mechanical stirring. Sodium chloride precipitates immediately. Stir and reflux for two and one-half to three hours and stop heating at the first sign of definite darkening. Remove the solvent at 50° under 12–15 mm. pressure and treat the yellow, semisolid residue with alkaline water. Extract the base into several liberal portions of ether, combine the ether extracts, and wash them with several portions of 5% sodium hydroxide solution and with water until all iodinated sodium phenolates have been removed. Dry the ether solution over anhydrous sodium sulfate, filter, and evaporate the solvent at 35–40°, carefully avoiding overheating the residual pale yellow oil.

Dissolve the oily base in good acetone without delay, and neutralize with absolute ethanolic hydrogen chloride to Congo-blue reaction. The salt (B-9) crystallizes soon. If necessary, some ether may be added to induce crystallization.

Filter the drug, wash it with a mixture of acetone and ether, and recrystallize from methanol and a little acetone four to six times without prolonged warming. The last mother liquors must be colorless. Wash the salt with acetone and finally with ether. The colorless crystals melt at 196° (decomp.) without preceding sintering. The yield varies from 40–50%.

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The Use of Acetylation in the Quantitative Separation of Ephedrine from Procaine*

By LLEWELLYN H. WELSH

Aqueous solutions of ephedrine give practically quantitative yields of the N-acetyl derivative by the action of acetic anhydride in the presence of excess bicarbonate. Under the same conditions procaine appears to acetylate at its primary amino group to yield a product which is basic by virtue of its tertiary nitrogen. The wide difference in properties of the acetyl derivatives of the two drugs permits their separation by simple extraction procedures, and the gravimetric determination of ephedrine as its acetyl derivative. The presence of small amounts of bisulfite greatly hinders the acetylation of ephedrine. The presence of ephedrine does not interfere in the determination of procaine by bromination.

RECENT WORK in this laboratory (1) established that the reaction of ephedrine with acetic anhydride at 70° yields the neutral N-acetyl derivative (hydroxyamide, I), m. p. 86–87°, and that the corresponding O-acetyl compound (aminoester, II) is capable of only a transitory existence before quantitatively rearranging into the hydroxyamide.

$$C_6H_5CHOHCH(CH_3)N(CH_3)COCH_3$$
(I)

$$C_6H_5CH(OCOCH_3)CH(CH_3)NHCH_3$$
(II)

These facts suggest the possibility of using acetylation in the analysis of drug mixtures containing certain aminoalcohols. If acetylation occurs quantitatively under conditions similar to those employed for the conversion of phenetedin to acetophenetedin (2), it should be possible to separate ephedrine and, perhaps, other aminoalcohols having an $ArCHOHCNH$ grouping from compounds containing unacetylatable basic (e.g., tertiary amino) groups.

It was found that acetylation of ephedrine at room temperature occurs rapidly in aqueous solution containing excess bicarbonate, and yields 98.0–99.4 per cent of the theoretical amount of acetyl derivative. The acetylation was then carried out on samples containing 150 mg. of ephedrine hydrochloride and 30 mg. of procaine hydrochloride. After acidification of the reaction mixture, the acetylephedrine was separated from the acetylated procaine salt by extraction with chloroform, and was determined gravimetrically after removal of the solvent. Recoveries of 97.6–99.2 per cent were obtained. When the procedure was applied to a commercial product purporting to contain in each cc. 50 mg. of ephedrine hydrochloride, 10 mg. of procaine hydrochloride, and "not more than" 1.5 mg. of sodium bisulfite, the results indicated a shortage of 10–14 per cent of ephedrine salt. Total solids and chloride determinations, however, supported the label declaration. Accordingly, a solution was prepared which contained *ca.* 3 mg. of sodium metabisulfite per 100 mg. of ephedrine hydrochloride. When aliquots of the solution were subjected to the acetylation procedure, recoveries of approximately 53% resulted. The unacetylated ephedrine could be extracted with chloroform after rendering the aqueous phase alkaline with sodium hydroxide. If, however, before acetylating the bisulfite was destroyed by the addition of iodine-potassium iodide solution, normal recovery percentages of acetyl derivative were obtained. The reason for the interference of bisulfite in the acetylation of ephedrine is not apparent to the writer. The molecular proportions of bisulfite, ephedrine, acetic anhydride, and bicarbonate present in the acetylation of the prepared solution are approximately 1:32:450:1500, respectively. Whatever the interference mechanism, one molecule of bisulfite effectively

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blocks the acetylation of approximately fifteen molecules of ephedrine under the conditions employed.

Although procaine appears to acetylate at its primary amino group under the conditions used in the acetylation of ephedrine, the derivative obtained could not be induced to crystallize. Procaine was determined in the presence of ephedrine by extracting the two compounds from alkaline solution into chloroform, transferring them from this solvent into aqueous solution by shakeouts with dilute acid, adding a slight excess of standard bromide-bromate solution to the acid extract, and back-titrating with thiosulfate after adding potassium iodide. Under these conditions ephedrine did not interfere with the bromination even when present in amounts five times that of the procaine, and the bromination results were slightly better than 99 per cent of the theoretical. The determination of procaine by this method is considered to be expedient rather than ideal, since it gives no information concerning the nature of the material brominated.

EXPERIMENTAL

(Melting points are corrected.)

Acetylation Procedure.—Sodium bicarbonate (3.0 Gm.) was added to a separatory funnel containing *ca.* 150 mg. of ephedrine hydrochloride dissolved in 10 cc. of water. The funnel was swirled until the liquid was practically saturated with bicarbonate, and a total of 1 cc. of acetic anhydride was then added in three approximately equal portions. After each addition of anhydride the funnel was securely stoppered and shaken vigorously. From time to time the shaking was interrupted and the stopcock was opened to release internal pressure. A fresh portion of anhydride was not added until carbon dioxide evolution resulting from the previous addition had practically ceased, and after each reaction small amounts of fluid in the stem and on the stopper and mouth of the funnel were rinsed back into the main body of liquid with a few drops of water. The reaction mixture was allowed to stand fifteen minutes after effervescence resulting from the last addition of anhydride apparently had ceased. It was then made acid to Congo red with 10% (*w/v*) sulfuric acid (about 20 cc. was required), an additional 5 cc. was added, and the acetylephedrine was extracted immediately with five 15-cc. portions of chloroform. Each extract was shaken in another funnel with a solution of 0.5 Gm. of sodium bicarbonate in 6 cc. of water to remove acetic acid before filtering through a pledget of cotton into a beaker. The filtered extracts were concentrated to a small volume, transferred with chloroform to a tared 50-cc. beaker (previously dried at 105° and cooled in a desiccator), and the remainder of the solvent removed on the steam bath in a current of air. The residual resin of acetylephedrine was heated thirty minutes in an oven at 105°, cooled in a desiccator, and weighed rapidly. Although crystalline anhydrous acetylephedrine does not tend to gain weight in the air under ordinary conditions, the supercooled substance does appear to gain slightly possibly because of a tendency to form the monohydrate (3). The weight of anhydrous residue multiplied by 0.9731 represents the equivalent of ephedrine hydrochloride. Residues of acetylephedrine crystallized slowly when seeded and triturated. The substance is not appreciably volatile at 105°, since a 500-mg. sample suffered an apparent loss of only 0.5 mg. when left thirty minutes in an oven at this temperature.

When bisulfite was present it was removed prior to the introduction of bicarbonate by swirling the funnel and adding, dropwise, a solution of 0.5 Gm. of iodine and 1.5 Gm. of potassium iodide in 25 cc. of water. After a slight excess of iodine had been added, a few drops of starch solution were introduced, and the blue color was discharged by addition of the necessary amount of 0.1 *N* sodium thiosulfate.

Table I lists recoveries obtained when the acetylation was carried out on solutions containing ephedrine hydrochloride (150 mg.) only, and solutions containing, in addition, procaine hydrochloride (30 mg.) and sodium metabisulfite (*ca.* 4.5 mg.). Melting points of various acetylephedrine residues are also listed.

TABLE I

Compounds Added	% Recovery	M. P. of Acetylephedrine
None	99.4	84.5–86°
	98.0	
Procaine	98.6	
	97.7	
	99.2	85 –86°
	97.6	
Bisulfite	51.7[a]	84.5–86°
	53.7[a]	
	97.9[b]	
	98.8[b]	85 –86°
Procaine and bisulfite	49.8[a]	85 –86°
Av.	98.4	

[a] Not included in average.
[b] Bisulfite removed with iodine before acetylation.

After acetylephedrine had been extracted from one of the reaction mixtures containing bisulfite, the aqueous solution was rendered alkaline and extracted several times with chloroform. Addition of hydrogen chloride and subsequent evaporation of solvent yielded ephedrine hydrochloride, m. p.

217–218°, equivalent to 97.2% of the ephedrine which had escaped acetylation.

When the procedure (modified in that extraction was made from alkaline solution) was applied to a 150-mg. sample of procaine hydrochloride, a 100.3% recovery of acetylprocaine was obtained. The colorless, resinous material showed a neutralization equivalent of 285 (calcd. 278.3 for $C_{15}H_{22}N_2O_3$). Hazard and Corteggiani (4) have reported a melting point of 74° for acetylprocaine, but material prepared in this laboratory could not be obtained in a crystalline condition. After the extraction of acetylephedrine from acidified reaction mixtures also containing acetylprocaine, the latter substance was extracted into chloroform after basifying the aqueous phase with sodium hydroxide. The approximately 30-mg. residues of acetylprocaine obtained after solvent removal amounted to 90–103% of the calculated quantity.

Determination of Procaine.—One cubic centimeter of 20% sodium hydroxide was added to 10 cc. of a solution containing 100 mg. of procaine hydrochloride, 500 mg. of the corresponding ephedrine salt, and 15 mg. of sodium metabisulfite. The alkaline solution was extracted with six 10-cc. portions of chloroform. The combined chloroform extracts were shaken with two 10-cc. portions of 5% sulfuric acid, then with four 10-cc. portions of water. The acid solution of ephedrine and procaine sulfates was washed with 5 cc. of petroleum ether and drained into a flask. The petroleum ether was then washed three times with 10-cc. portions of water which were added to the contents of the flask. The acid solution was allowed to stand, with occasional shaking, until the odor of petroleum ether had practically disappeared and no fine droplets of the solvent could be observed. The volume was then adjusted to about 150 cc. by the addition of water and 9 cc. of conc. hydrochloric acid, and two drops of 0.1% methyl red indicator were added. The solution was brominated essentially as described by Wells (5). Tenth-normal bromide-bromate solution was added from a burette at a rate of approximately two drops per second while the contents of the flask were constantly agitated. The indicator added originally faded gradually during the bromination, and an additional one or two drops were necessary before the end of the titration. As the anticipated end point was approached the speed of addition of brominating reagent was reduced and the effect of one-drop additions was observed. The approximate end point was reached when the indicator suddenly became decolorized, and a faint odor of bromine was noticeable. About 1 Gm. of potassium iodide was then dissolved in the solution, and back-titration with 0.1 *N* sodium thiosulfate was effected in the presence of starch. The required thiosulfate varied between 0.1 and 0.2 cc. Results of determinations ranged from 99.2% to 99.4% of the theoretical. A blank determination carried through the several stages described above, but in the absence of procaine, resulted in the consumption of only 0.03 cc. of bromide-bromate solution.

SUMMARY

Aqueous solutions of ephedrine give practically quantitative yields of the N-acetyl derivative by the action of acetic anhydride in the presence of excess bicarbonate. Under the same conditions procaine appears to acetylate at its primary amino group to yield a product which is basic by virtue of its tertiary nitrogen. The wide difference in properties of the acetyl derivatives of the two drugs permits their separation by simple extraction procedures, and the gravimetric determination of ephedrine as its acetyl derivative. The presence of small amounts of bisulfite greatly hinders the acetylation of ephedrine.

The presence of ephedrine does not interfere in the determination of procaine by bromination.

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The Synthesis and Study of Some N-Dihydroxypropyl Derivatives*

By JAMES W. JONES† and ALFRED HALPERN‡§

The N-dihydroxypropyl derivatives of sulfathiazole, saccharin, and phenobarbital have been prepared by the reaction between both the sodium and the silver salts of the imides with glyceryl-α-chlorhydrin. Attempts at crystallization from various solvents failed to yield crystalline material. The influence of silver hydroxide and copper powder on the yield of product from the above reaction was determined The solubilities of the dihydroxypropyl derivatives in water were studied.

RECENTLY it was shown that the introduction of the dihydroxypropyl group in theophylline did not change the therapeutic properties of the compound and led to a stable, water-soluble derivative (1). It became of interest, therefore, to determine the effect on the solubility by the introduction of the dihydroxypropyl group in other nitrogen compounds difficulty soluble in water.

Several compounds of therapeutic importance, of which an acid-stable water-soluble derivative would be advantageous, were chosen for the study. Sulfathiazole (a representative "sulfa" drug), saccharin, and phenobarbital (a typical barbiturate) were the imides selected for N-dihydroxypropyl substitution. These compounds are all soluble in dilute alkali. However, their alkali salts are probably decomposed to liberate the water-insoluble base in the gastric fluids as was shown for the sodium salt of theophylline (1).

In the case of the "sulfa" drugs a stab e water-soluble derivative would prevent crystallization in the urinary tract. A soluble saccharin derivative would be of value in preparing aqueous pharmaceuticals of acid reaction. A neutral, water-soluble barbiturate would greatly enhance the parenteral administration of these drugs.

The N-hydroxyethyl derivative of phenobarbital has been prepared by the reaction between the sodium salt of phenobarbital and ethylenemonochlorhydrin (2). A huge excess of the ethylenemonochlorhydrin was used and the N-derivative was obtained in fairly good yields. A modification of this method was used as a general method for the preparation of the N-dihydroxypropyl derivatives.

EXPERIMENTAL

Method A.—To 0.1 mole of the sodium salt of the imide, in a 3-neck flask fitted with a stirrer and a thermometer, was added 1.5 mole of glyceryl-α-chlorhydrin. The mixture was heated on an oil bath for two hours at a temperature of 120–130°. Rapid stirring was maintained throughout the reaction period. At the end of this time, the reaction mixture was cooled, and the stirring stopped. The mixture was filtered by suction and the residue on the filter paper washed with two 20-cc. portions of hot absolute ethanol. The residue on the filter paper, which was identified as sodium chloride, weighed 5.2 Gm. The theoretical quantity was 5.8 Gm. The excess glyceryl-α-chlorhydrin and the ethanol were removed by distillation under reduced pressure. The residue was a brown, gummy material.

The residues were purified through repeated precipitation by ether from absolute ethanol. After four such precipitations, a colorless oil was obtained, which, after the removal of the last traces of the solvent under reduced pressure, hardened to a plastic semisolid resin. The yield of purified derivative was:

	Yield		
	Found, Gm.	Theoretical, Gm.	%
(I) N-Dihydroxypropyl-sulfathiazole	20.4	31.4	65
(II) N-Dihydroxypropyl-phenobarbital	19.8	29.1	68
(III) N-Dihydroxypropyl-saccharin	16.5	24.2	68

% Nitrogen (Kjeldahl)
Calc'd.: (I) 13.37, (II) 9.62, (III) 7.64
Found : (I) 13.47, (II) 9.71, (III) 7.41

Method B.—One-tenth mole of the sodium salt of the imide was dissolved in 300 cc. of distilled water and heated to boiling. Five-tenths mole of glyceryl-α-chlorhydrin was added and the solution boiled for two hours, water being added from time

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to time to maintain the volume. The water was then removed under reduced pressure and the residue extracted with five 50-cc. portions of hot absolute ethanol. The sodium chloride remaining weighed 4.8 Gm. The ethanol was removed by distillation under reduced pressure and the residue purified in the above manner. The products of these reactions were also plastic semisolid materials. The yield of product obtained:

	Yield Found, Gm.	Yield Theoretical, Gm.	%
(I) N-Dihydroxypropyl-sulfathiazole	12.5	31.4	40
(II) N-Dihydroxypropyl-phenobarbital	12.2	29.1	42
(III) N-Dihydroxypropyl-saccharin	9.9	24.2	41

% Nitrogen (Kjeldahl)
Calc'd.: (I) 13.37, (II) 9.62, (III) 7.64
Found : (I) 13.26, (II) 9.42, (III) 7.40

Method C.—Substituting the silver salt for the sodium salt in both reactions (A and B) resulted in products identical to those obtained from the sodium salts. A quantitative yield of silver chloride was obtained. The yield of N-dihydroxypropyl imide obtained from the silver salt in Method A was:

	Yield Found, Gm.	Yield Theoretical, Gm.	%
(I) N-Dihydroxypropyl-sulfathiazole	25.7	31.4	82
(II) N-Dihydroxypropyl-phenobarbital	24.3	29.1	84
(III) N-Dihydroxypropyl-saccharin	20.0	24.2	83

% Nitrogen (Kjeldahl)
Calc'd.: (I) 13.37, (II) 9.62, (III) 7.64
Found: (I) 13.41, (II) 9.60, (III) 7.58

The yield of N-dihydroxypropyl imide obtained from the silver salt in Method B was:

	Yield Found, Gm.	Yield Theoretical, Gm.	%
(I) N-Dihydroxypropyl-sulfathiazole	23.8	31.4	76
(II) N-Dihydroxypropyl-phenobarbital	23.2	29.1	80
(III) N-Dihydroxypropyl-saccharin	18.1	24.2	79

% Nitrogen (Kjeldahl)
Calc'd.: (I) 13.37, (II) 9.62, (III) 7.64
Found: (I) 13.40, (II) 9.56, (III) 7.54

Preparation of the Silver Salt of the Imides (3).—One-tenth mole of the sodium salt of the imide was dissolved in 300 cc. of distilled water. The solution was stirred with a mechanical stirrer and a solution of 0.1 mole of silver nitrate in 200 cc. of distilled water was added. An immediate precipitation occurred and the mixture was stirred for fifteen minutes longer. The precipitate was filtered off on a Büchner funnel and washed well with distilled water, then dried in a vacuum desiccator overnight. The silver salts must be protected from light to prevent darkening of the compound. The yield of silver imide obtained was:

	Yield Found, Gm.	Yield Theoretical, Gm.	%
Silver sulfathiazole	33.5	36.3	.90
Silver phenobarbital	36.7	39.9	92
Silver saccharin	27.6	29.0	95

Method D.—Reactions A and B were repeated with the addition of silver hydroxide and copper powder as catalysts. One-half gram of freshly precipitated AgOH was added to the reaction mixture and the process carried out in the indicated manner. The average yield of substituted imide was increased. The yield of compound obtained from Method A catalyzed by silver hydroxide was:

	Yield Found, Gm.	Yield Theoretical, Gm.	%
N-Dihydroxypropyl-sulfathiazole	23.1	31.4	74
N-Dihydroxypropyl-phenobarbital	22.9	29.1	79
N-Dihydroxypropyl-saccharin	18.8	24.2	78

The yield of compound obtained from Method B catalyzed by silver hydroxide was:

	Yield Found, Gm.	Yield Theoretical, Gm.	%
N-Dihydroxypropyl-sulfathiazole	22.2	31.4	71
N-Dihydroxypropyl-phenobarbital	21.1	29.1	73
N-Dihydroxypropyl-saccharin	17.4	24.2	72

Copper powder was used in a similar manner and the yields in this instance were increased, though not as high as with silver hydroxide. The yield of compound obtained from Method A catalyzed by copper powder was:

	Yield Found, Gm.	Yield Theoretical, Gm.	%
N-Dihydroxypropyl-sulfathiazole	21.6	31.4	69
N-Dihydroxypropyl-barbital	20.9	29.1	72
N-Dihydroxypropyl-saccharin	16.9	24.2	74

The yield of compound obtained from Method B catalyzed by copper powder was:

	Yield Found, Gm.	Yield Theoretical, Gm.	%
N-Dihydroxypropyl-sulfathiazole	20.0	31.4	64
N-Dihydroxypropyl-phenobarbital	19.1	29.1	66
N-Dihydroxypropyl-saccharin	16.2	24.2	67

N-Allylphenobarbital was prepared using 0.1 molar quantities from ethyl phenyl-ethyl malonic ester and allyl urea in the presence of sodium methylate and methyl alcohol. The yield was 63% of the theoretical. Melting point found: 67–68°. Recorded melting point: 68–69° (4).

The dihydroxypropyl derivative of phenobarbital was obtained by the oxidation of N-allylphenobarbital with cold, dilute, aqueous potassium permanganate. A product identical to that obtained by the previous reactions was obtained. The yield was 52% of the theoretical. Nitrogen calculated: 9.62. Nitrogen found: 9.31.

Attempts at crystallizing the dihydroxy derivatives from acetone, acetone-benzene, benzene, benzene-ligroin, alcohol, aqueous alcohol, alcohol-ether, and water solutions failed. The product obtained in all cases was a plastic, semisolid resin.

It was of interest to note that hydroxymethylene-bis(1-methylenephenobarbital) prepared by the reaction between glyceryl α-γ dibromohydrin and phenobarbital in the presence of sodium in methanol was reported as a hard glassy solid without definite melting point (2). Furthermore, the gum obtained from this reaction could not be induced to crystallize from any of the usual organic solvents.

The substituted compounds are stable at room temperature. They decompose on heating above 180°. Attempts at distillation of the compounds at 2 mm. Hg pressure resulted in decomposition of the products.

The N-dihydroxypropyl derivatives all gave a positive periodic acid test (5) for the glycol group.

The compounds were soluble in alcohol, slightly soluble in acetone, and insoluble in ether, chloroform, and petroleum ether. The solubility of the compounds in water at 25° was found to be as follows:

N-Dihydroxypropyl-sulfathiazole	1 Gm. in 330 cc.
N-Dihydroxypropyl-phenobarbital	1 Gm. in 200 cc.
N-Dihydroxypropyl-saccharin	1 Gm. in 90 cc.

The introduction of the N-dihydroxypropyl or N-ethylhydroxy group in saccharin resulted in a tasteless compound.

The solubility of the compounds in water was increased by the introduction of the dihydroxypropyl group. The noncrystalline nature of the compounds, however, greatly diminished the advantages of solubility thus obtained. The rather lengthy purification procedures necessary to obtain a pure compound also detracted from the increased solubility advantage. Further study of these compounds was discontinued for the above reasons.

SUMMARY AND CONCLUSIONS

1. The effect on solubility in water by the substitution of the dihydroxypropyl group in difficultly soluble imides was studied.

2. The N-dihydroxypropyl derivatives of sulfathiazole, saccharin, and phenobarbital were prepared by the reaction between the sodium salt of the imide and glyceryl-α-chlorhydrin. The compounds were also prepared from the silver salts of the imide and glyceryl-α-chlorhydrin.

3 The products were all plastic, semisolid, resinous materials. Attempts at crystallization from various solvents failed to yield a crystalline material.

4. The influence of silver hydroxide and copper powder on the yield of product from the above reaction was noted.

5. N - Dihydroxypropylphenobarbital prepared by the oxidation of N-allylphenobarbital with potassium permanganate was identical to that obtained by the previous means.

6. The solubility of the dihydroxypropyl derivatives in water was determined.

7. The study was discontinued because the difficulties in obtaining a pure compound and the plastic semisolid resinous state detracted from the pharmaceutical utility of the crystalline parent compounds. The advantages of increased solubility did not compensate for the above disadvantages.

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Chemical Determination of Synthetic Estrogens
I. Basic Data on the Nitrosophenol Reaction*

By SIDNEY GOTTLIEB

A method is described for the identification and quantitative determination of diethylstilbestrol, hexestrol, dienestrol, benzestrol, and Meprane which depends on the formation of the corresponding *o*-nitrosophenols. The absorption of the alkaline quinoid forms of these derivatives in solution is a linear function of the concentration.

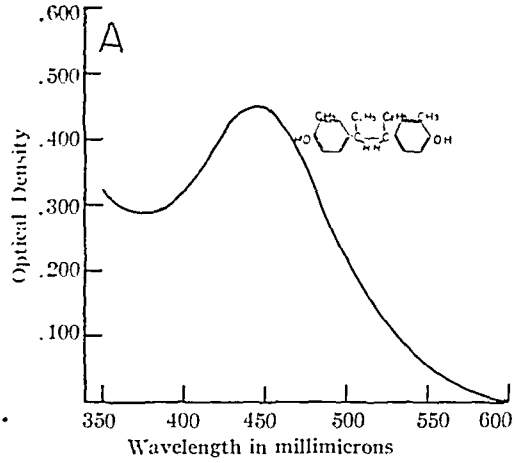
Dodds, *et al.* (1), reported the extremely high estrogenic activity of the 4,4′-stilbenediols in 1938. Since then this group of compounds has come into wide and important use in the treatment of various disorders stemming from estrogen deficiency. Virtually each year sees a new derivative of this series put on the market. A reliable and widely applicable method for the determination of these compounds is needed; it is the purpose of this contribution to propose such a method and to present the basic data on it.

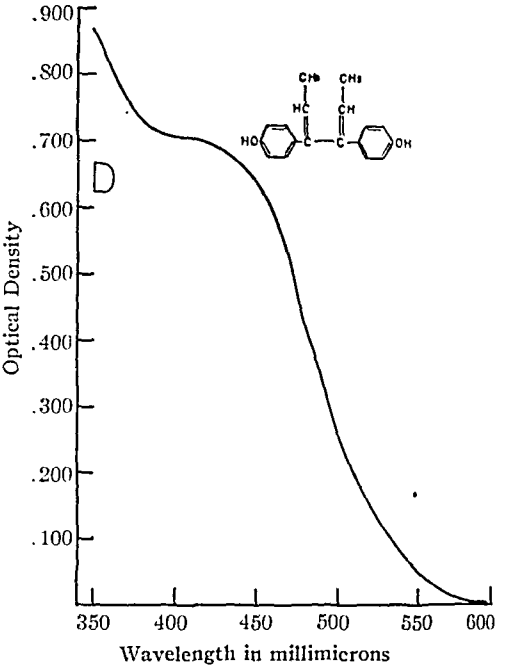
Various methods have been proposed for the chemical determination of compounds in the 4,4′-stilbenediol series. Elvidge (2) and Goetz and Seif (3) proposed absorption spectrophotometry of the unaltered compounds for diethylstilbestrol, diethylstilbestrol dipropionate, and hexestrol. Dingemanse (4) determined diethylstilbestrol by its reaction with antimony pentachloride to form a red addition compound. The color was reported to be relatively unstable and fats, sterols, and colored substances interferred with the reaction. Tubis and Bloom (5) suggested the use of the Folin and Ciocalteu reagent (molybdo-phosphotungstic acids) for the determination of diethylstilbestrol in tablets. This method has been included in the U. S. P. XIII for diethylstilbestrol capsule, injection, and tablet preparations. Since the reaction depends on the reduction of the labile phosphomolybdic-phosphotungstic acids to the blue oxides of molybdenum and tungsten, its use is limited in many types of preparations. The coupling of diethylstilbestrol with diazobenzenesulfonic acid to form a colored solution has been used by Huf and Widmann (6) as an assay for this estrogen. An accuracy of 10 per cent is claimed for the method and the diazotization to produce the reagent must be carried out daily. A bromometric procedure for determining 1–40 mg. of diethylstilbestrol has been described by Sondern and Burson (7) and the reaction of various stilbenediols with bromine in acetic acid has been used by Cocking (8) as a colorimetric assay procedure. Malpress (9) has elaborated a method depending on the nitration of diethylstilbestrol, hexestrol, and dienestrol to polynitro compounds and the subsequent measurement of the orange color produced by these nitro derivatives in alkaline solution. A determination of dienestrol in biological samples has been proposed by Smith (10) utilizing the formation and subsequent titration of the maleic acid adduct. Recoveries of 60–80 per cent of added dienestrol from urine are reported.

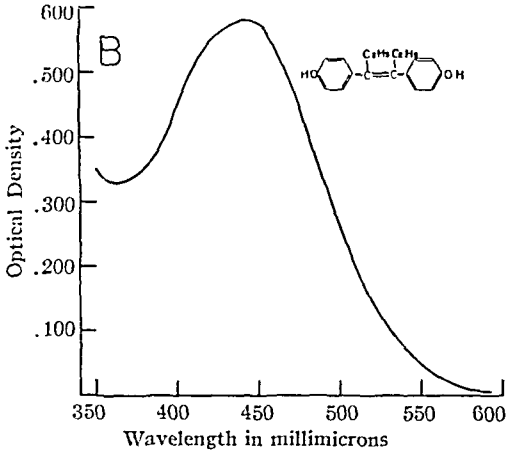
In this paper the author reports a method depending on the formation of nitrosophenols from various synthetic estrogens. The method affords an opportunity to distinguish among individual members of this group of compounds and to determine each compound quantitatively. In a review of color tests for phenols, Gibbs (15) discussed the formation of nitrosophenols and their quinone oximes. Stoughton (11) published a method for the estimation of phenols in biological materials which consisted of treating the phenol with nitric and sulfuric acids at 100° to form a nitrosophenol which rearranges in the presence of excess alcoholic ammonium hydroxide to form a highly colored quinoid compound.

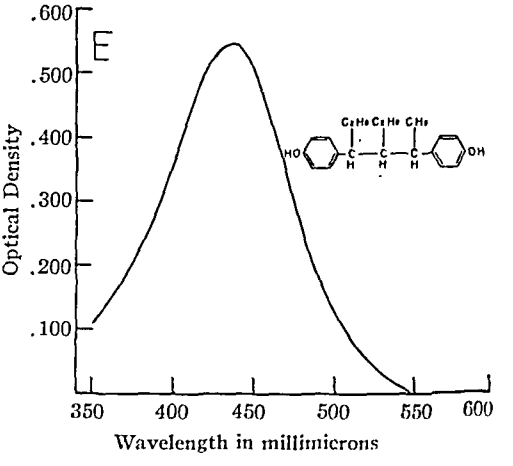
Wetlaufer, *et al.* (12) modified this procedure slightly to determine traces of phenols in hydrocarbon solvents and Lykken, *et al.* (13) made some further modifications in

* Received July 24, 1947, from the Chemical Section, Medical Division, Food and Drug Administration, Federal Security Agency, Washington, D. C.









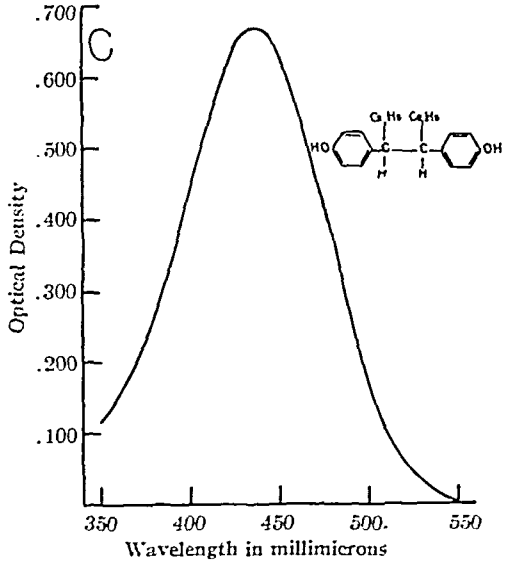
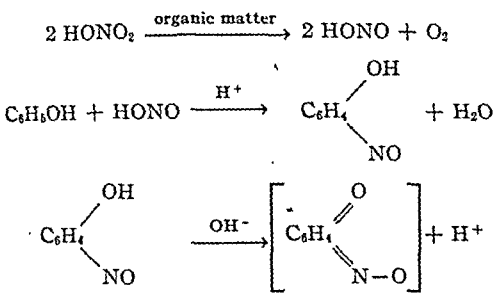


Figure 1 (A – E)

Absorption curves for nitrosophenol quinoid compounds from five synthetic estrogens.

A—phenol from Meprane
B—diethylstilbestrol
C—hexestrol
D—dienestrol
E—benzestrol

using the reaction for the same purpose. Using phenol as an example, the probable reactions are as follows:



The method described below has been applied to derive data on absorption and calibration curves for five important synthetic estrogens, namely diethylstilbestrol (3,4-bis - (*p* - hydroxyphenyl) - hexene - 3), hexestrol (3,4-bis-(*p*-hydroxyphenyl) hexane), dienestrol (3,4-bis-(*p*-hydroxyphenyl) hexadiene-2,4), benzestrol (2,4-bis-(*p*-hydroxyphenyl)-3-ethylhexane), and Meprane (3,4-bis-(*m*-methyl-*p*-propionoxyphenyl)-hexane). A method for the application of this reaction to pharmaceutical preparations has been worked out and is at present being collaboratively checked by several laboratories. It will be the subject of a subsequent publication.

EXPERIMENTAL

Reagents.—Buffer solution: To 800 cc. of glacial acetic acid, add 150 cc. of 10% potassium hydroxide solution and 50 cc. of water, and mix well.

Saturated aqueous sodium nitrite solution.

Concentrated sulfuric acid (36 *N*).

Alcoholic ammonium hydroxide solution. Mix 450 cc. anhydrous ethyl alcohol or isopropyl alcohol, 300 cc. of conc. ammonium hydroxide (14 *N*), and 250 cc. of water.

Method.—Make up a standard solution of the compound being investigated by dissolving 10 mg. in 100 cc. of 95% ethyl alcohol. For diethylstilbestrol, hexestrol, dienestrol, and benzestrol, make up a series of concentrations in the range of 0.00 to 1.00 mg. by evaporating the requisite amount of alcoholic solution in 100-cc. beakers. In the case of the phenol from Meprane, the range should be from 0.0 to 2.0 mg. The residue in each beaker is dissolved in 5 cc. of buffer solution; gentle warming may be necessary to completely dissolve several of the compounds. Five drops of concentrated sulfuric acid is then added, followed by 2 drops of saturated sodium nitrite solution. The solution is allowed to stand at room temperature for thirty minutes with occasional shaking. The solution is then transferred quantitatively to a 50-cc. volumetric flask and diluted to near the mark with alcoholic ammonium hydroxide solution, cooling the flask in an ice bath as the alkali is added, and using the alkali to wash out the beaker. Let stand thirty minutes, dilute exactly to the mark with alcoholic ammonium hydroxide solution, and filter through a dry filter. This filtered solution is then used to make the photometric measurements. The color is stable for at least several hours after filtration.

Hydrolysis of Meprane.—The free phenol was obtained in quantitative yield from its dipropionic acid ester by refluxing about 0.5 Gm. of the ester in a 10% methanolic solution of potassium hydroxide for one hour. The methanolic solution was cooled, diluted with 100 cc. of distilled water, acidified with 10% HCl, and quantitatively transferred to a separatory funnel. This solution was then extracted four times with 50-cc. portions of peroxide-free diethyl ether. The combined ether extracts were washed three times with 100-cc. portions of 5% sodium bicarbonate and twice with 100-cc. portions of water. The ether solution was then separated, filtered through a dry filter, the filter washed with ether and the ether solution evaporated to dryness. The phenol (3,4-bis-(*m*-methyl-*p*-hydroxyphenyl)-hexane) melted at 142–145° (corr.). Ten milligrams of this material was dissolved in 100 cc. of 95% ethyl alcohol to make up the standard solution.

Photometric Measurements.—The absorption curves in Fig. 1 were obtained by treating 1 mg. of each compound as described above under "Method," and studying the absorption of the solutions so obtained in the spectral range of 350 to 600 mμ. The instrument used was a Beckmann Model DU Quartz Spectrophotometer, with the blue-sensitive phototube in position. The standard calibration curves shown in Fig. 2 were obtained by measuring the absorption of each sample at a wave length of 440 mμ on the Beckmann instrument. For both the absorption and calibration curves, the instrument was set at 100% transmission (or zero optical density) for a tube containing a blank solution which had been run through the entire procedure, omitting the addition of any phenolic compound.

Stability of Color.—Lykken, *et al.* (13), report that the colors produced by nitrosophenol quinoid compounds slowly increase in intensity, and these investigators recommend allowing the solutions to stand overnight before measuring the color. The synthetic estrogens apparently do not show this instability when treated as described above. Table I portrays a typical experiment with diethylstilbestrol, indicating no appreciable fading over a period of four hours. The other estrogens behaved in a similar manner.

TABLE I.—STABILITY OF SOLUTIONS OF THE NITROSOPHENOL QUINOID FROM DIETHYLSTILBESTROL. VARIOUS AMOUNTS OF DIETHYLSTILBESTROL TREATED AS DESCRIBED UNDER "METHOD"

Diethylstilbestrol	Optical Densities of Solutions at —Various Times after Development of Color—				
Mg.	15 Min.	30 Min.	1 Hr.	2 Hr.	4 Hr.
0.1	0.051	0.060	0.062	0.060	0.061
0.5	0.280	0.295	0.293	0.295	0.294
1.0	0.509	0.580	0.580	0.575	0.579

DISCUSSION

It can be seen from Fig. 1 that the absorption curves of the quinoid compounds formed by the estrogens studied are sufficiently different to enable their identification in a preparation containing only one estrogen. The accurate quantitative determination of one in the presence of another would be possible only in the case of a hexestrol-dienestrol or a benzestrol-dienestrol mixture, but the simultaneous determination of other pairs might be possible if a study of the absorption of the quinoid compounds in the region below 350 mμ was made. At any rate, it is improbable that mixtures of these compounds would occur in pharmaceutical preparations.

The natural estrogens, estrone, and α-estradiol have been submitted to this procedure and their absorption at 440 mμ (Table II) was found to be so low in comparison to the synthetic estrogens that their contribution to the absorption in mixtures of synthetic and natural estrogens can be ignored, or, in very careful work, easily corrected for by a separate determination (14).

TABLE II.—OPTICAL DENSITY OF SOLUTIONS OF THE NITROSOPHENOL QUINOIDS FROM ESTRONE, α-ESTRADIOL, AND DIETHYLSTILBESTROL. ONE MILLIGRAM OF EACH COMPOUND TREATED AS DESCRIBED UNDER "METHOD"

Compound	Optical Density
Estrone	0.020
α-Estradiol	0.044
Diethylstilbestrol	0.580

It will be noticed that in the case of the phenol 3,4-bis(*m*-methyl-*p*-hydroxyphenyl)-hexane, the optical density is appreciably less than that of the other estrogens in comparable concentrations. This is undoubtedly due to the hindering effect of the methyl group in the position ortho to the phenolic group. The presence of these methyl groups probably effectively blocks two of the four possible sites for the introduction of the nitroso group in the molecule.

Although this method is specific for phenolic compounds, it will not distinguish among several phenolic compounds, unless the spectral characteristics of the quinoid compounds formed had been previously studied. However, as mentioned above, other phenols are usually not encountered in pharmaceutical preparations containing these estrogens.

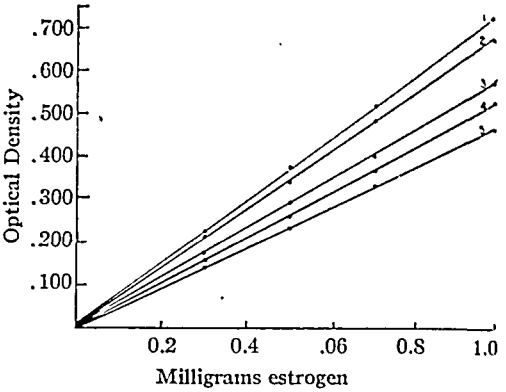


Fig. 2.—Standard calibration curves for the nitrosophenol quinoid compounds from five synthetic estrogens. 1—dienestrol; 2—hexestrol; 3—diethylstilbestrol; 4—benzestrol; 5—phenol from meprane.

SUMMARY

Basic data are presented on a new chemical method for the determination of the synthetic estrogenic compounds, 3,4-bis-(*p*-hydroxyphenyl)-hexene-3, 3,4-bis-(*p*-hydroxyphenyl)-hexane, 3,4-bis-(*p*-hydroxyphenyl)-hexadiene-2,4, 2,4-bis-(*p*-hydroxyphenyl)-3-ethylhexane, and 3,4-bis-(*m*-methyl-*p*-propionoxyphenyl)-hexane. A study of the absorption curves of the alkaline quinoid form of the ortho-nitrosophenols derived from these estrogens by a simple procedure indicates that they can be qualitatively identified by this means. Calibration curves are presented to indicate the correspondence of concentration and color in this reaction. The natural estrogens, estrone, and α-estradiol, do not interfere.

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Comparative *In Vitro* Neutralization of Heparin*

By E. J. MACKOWIAK and R. D. BARNARD

Four classes of antiheparinics have been examined for the *in vitro* restoration of spontaneous coagulability to heparinized human blood. Of the four antiheparinics studied, toluidine blue was found to be more effective than modified human globin, hematoxylin, or lactose.

THE HISTONE, protamine, has been used to neutralize the anticoagulant effect of previously administered heparin (1–3). Occasion for such use has seldom arisen and suitable pharmaceutical protamine preparations are consequently sparse. A broader significance of antiheparinic therapy was recently uncovered when it was discovered that bleeding in a certain category of hemorrhagic dyscrasias was due to an autochthonous increase of heparin in the blood. Volkert and Hertel (4) found heparin in identifiable and determinable quantities in the blood of acute leukemics and Barnard and Sprinz (5), in their series of acute leukemic patients, found that 40 per cent of deaths were due to heparinemic hemorrhage into a vital organ. It was furthermore found that the heparinemia in this condition, as in the hemorrhagic phases of thrombopenia, aplastic anemia, agranulocytosis, anaphylaxis, and radiation sickness was preceded by a failure of erythroid marrow function and a reduction in blood cholinesterase (6). This sequence—the atopic-exudative syndrome (7)—is rather more common in clinical medicine than is usually appreciated. Barnard and Sprinz (5) were able to restore the coagulability in the blood of a patient with terminal acute leukemia by the addition of the histone, modified human globin.

The emergence of antiheparinic therapy from its present role as an occasional procedure in correcting the effects of an overadministration of one medicinal agent with another, to that of its potentially greater role as a life-saving procedure in a wide category of hemorrhagic dyscrasias and the possible broadening of the scope of such therapy should radiation sickness develop into an industrial or a wartime hazard, led us to undertake a search for additional antiheparinics. Four classes of substances with antiheparinic potency have been investigated and will be discussed from a standpoint of possible therapeutic applicability. This discussion is based on the *in vitro* measurement of antiheparinic potency; the order of discussion is the chronology of investigation.

I. Modified Human Globin.—The material used by us was that developed as an oncotically active colloid by Dr. M. M. Strumia and his co-workers at the Bryn Mawr Hospital in Pennsylvania. Samples which had proved to be pyrogenic on human administration as well as nonpyrogenic samples proved to be equally effective in restoring spontaneous coagulability to heparinized human blood. The action is rather slow, about twelve hours being required for complete heparin neutralization regardless of the initial concentration of the latter. The presence of 400 mg. % of globin is necessary to vitiate 2 mg. % of heparin. Such a concentration of globin is readily attained clinically and 2 mg. % of heparin is the highest concentration that has been encountered in any instance of estrapenic shock. The slowness of action of globin, however, would seem to pose a theoretic disadvantage which might be offset by its relatively low toxicity and the future ready availability of the material with the re-advent of a National Blood Donor Program.

II. Toluidine Blue.—Allen and Jacobson (8) used toluidine blue to restore coagulability to the blood of dogs in the hemorrhagic phase of radiation sickness and to humans with acute leukemia. Neutralization of heparin by toluidine blue has long been known (9); in fact this dye had been used to trace the origin of heparin to the basophilic granules of the mast cells of Ehrlich (10).

Our experiments fully confirmed the findings of Allen and Jacobson. Whereas heparinemia is not the sole determining factor in bleeding during an atopic-exudative reaction, neutralization of the heparinemia serves to arrest such bleeding even though the cholinergic intoxication of the atopic-exudative syndrome persists. It was found that toluidine blue would almost instantly neutralize heparin and restore coagulability to fluid blood containing as much as 100 mg. % of heparin. Toluidine blue is unquestionably the best antiheparinic (at least in its theoretic promise) that we have tested.

* Received August 12, 1947, from the Veterans Administration Hospital, Batavia, N. Y., and the Terrace Heights Hospital, Hollis, L. I., N. Y.

III. Hematoxylin.—Because hematoxylin also stains the basophilic granules in the mast cells and because it has proved nontoxic on oral administration (it has been used in doses as high as 5 Gm. as an astringent in diarrheas) the antiheparinic effect of this dye was tested *in vitro*. For concentrations up to 2% of heparin, it is equal in effectiveness to toluidine blue but for higher concentrations of the anticoagulant, the action of hematoxylin becomes complicated by a marked precipitation of plasma proteins.[1]

Whether the facility of oral administration and the cost of purification of logwood extracts will justify their exploitation as antiheparinics is a question that still remains to be decided.

IV. Polysaccharides.—Lactose, in solid phase, induces rapid coagulation of heparinized blood. Starch and glycogen have a similar, though weaker, effect. Solutions of polysaccharides containing no excess in solid phase are devoid of antiheparinic action. Presumably in this instance we are dealing with a surface reaction, the heparin being adsorbed by and its acid groups neutralized by the hydroxyls of the polysaccharide. There appears to be no chance for the pharmaceutic utilization of the heparin neutralizing properties of saccharides, at least in systemic antiheparinic therapy, and the phenomenon is included in the report only because of its possible academic interest.

[1] In this connection it must be pointed out that all electropositive colloid substances may precipitate certain fractions of the plasma proteins, including fibrinogen, i.e., they may in themselves be directly thromboplastic. Thus samples of globin and of protamine have been encountered which cause an immediate coagulation of plasma by protein precipitation and "pseudoclot" formation. The mechanism of this coagulation in the case of globin has been previously outlined (11). The fact that certain preparations of protamine may evoke a similar precipitation phenomenon leads to the conclusion that the apparently heightened coagulability of the blood of normal humans (12) cannot be interpreted as due to neutralization of the "normal" heparin content extant.

The excess protamine content of certain protamine-insulin and protamine-zinc-insulin preparations has been utilized by physicians for the control of capillary oozing even when the latter was probably not on the basis of hyperheparinemia. Such protamine-insulin preparations have a definite protein precipitant action. Modified human globin (Strumia, 13) which is devoid of such nonspecific protein precipitant action has been found not to affect the coagulation time of normal human subjects after intravenous administration.

SUMMARY AND CONCLUSIONS

Four pharmaceutic classes of antiheparinics have been tested for the *in vitro* restoration of spontaneous coagulability to heparinized human blood. The investigation was undertaken because of the prominent role which such therapy may assume in the hemorrhagic phases of atopic exudative syndromes such as the blood dyscrasias and radiation sickness.

In rapidity of action and *in vitro* effectiveness, toluidine blue is found to be superior to both hematoxylin and modified human globin.

Lactose, in solid phase, is antiheparinic but there appears to be no chance of the utilization of this property in practical antiheparin therapy.

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WHO MAKES IT?

The National Registry of Rare Chemicals, Armour Research Foundation, 33rd, Federal and Dearborn Streets, Chicago, Ill., seeks information on sources of supply for the following chemicals:

Ethyl acetamidomalonate
Thioindoxyl-2-carboxylic acid
Cholinesterase
Methylphenylbarbituric acid
2,2-Dichlorocamphane
3,4-Dihydroxynorephedrine
Creatinephosphate
Stevioside
N'-3,4-Dimethylbenzoylsulfanilamide
3-Carboxy-4-hydroxypyrazole
Corynanthine
Kephalin
d-Quercitol
Chondroitin sulfuric acid
β-(2-Pyrrole)acrylic acid
Quebrachitol
Indoxyl potassium sulfate
Dipeptidase
Nucleotidase
Porphyrexide
Hydroxytyramine

The Bactericidal Action of Oxygen-Liberating Substances on Oral Microorganisms. I. Studies with Fusiform Bacilli*

By THOMAS C. GRUBB and MARGUERITE A. EDWARDS

Two freshly isolated strains of fusiform bacilli, cultured in Brewer's thioglycollate medium, were inoculated into varying concentrations of sodium perborate, hydrogen peroxide, and a neutralized perborate dentifrice, diluted with water, saliva or saliva plus serum. Subcultures were made after thirty seconds, one, two, three, five, ten, and fifteen minutes' contact. The presence or absence of growth after seven days' incubation indicated the germicidal action of the substances tested. In a dilution of 1:5 the neutralized perborate dentifrice killed all the test organisms in one minute or less. After five minutes' contact the perborate dentifrice killed the test organisms in dilutions up to 1:40 in most instances. When sodium perborate and hydrogen peroxide were compared with the perborate dentifrice in various dilutions of equal oxygen content, hydrogen peroxide was more effective and sodium perborate less effective than the perborate dentifrice in the higher dilutions. Although all three products were equally effective in the lower dilutions usually employed for oral use, the strong alkalinity of sodium perborate and the high acidity of commercial hydrogen peroxide solutions are considered undesirable properties of products intended for oral application.

ALTHOUGH oxygen-liberating substances have been used for many years in the treatment of oral infections, especially Vincent's stomatitis, there appear to be few studies published on their bactericidal properties. Indeed, with regard to sodium perborate the United States Dispensatory (1) states, "It is rather extraordinary that despite the tremendous quantity of it which is used today, as far as we can find there is only one experimental investigation (2) concerning its bactericidal properties which has been published." Furthermore, so far as the writers are aware, no study has been published showing the *in vitro* action of oxygen-liberating compounds on the organisms associated with Vincent's infection—*Fusiformis dentium* and *Borrelia vincenti*. Possibly the reason for this situation is that fusiform bacilli and especially oral spirochetes are relatively difficult to isolate and culture so that they may be utilized for *in vitro* tests. Since the recent literature indicates methods for isolating and culturing fusiform bacilli (3) and oral spirochetes (4, 5) it now is practicable to determine the action of oxygenating agents on these organisms. The present report describes studies on the bactericidal action of hydrogen peroxide, sodium perborate, and a neutralized sodium perborate dentifrice[1] on fusiform bacilli.

* Received June 11, 1947, from the Research Laboratories, Vick Chemical Company, Flushing, N. Y.

EXPERIMENTAL

Two strains of fusiform bacilli (G. H. and F. N.) were isolated from normal, healthy mouths of two individuals by the method of Hine and Berry (3). These strains grew readily in Brewer's thioglycollate medium (6) and corresponded morphologically (see Fig. 1) and biochemically to *Fusiformis dentium* described by Hine and Berry. The number of fusiform bacilli in a given inoculum was readily determined by making a series of successively higher dilutions in Brewer's medium, incubating the tubes for twenty-four hours at 37° and counting the number of colonies in the tubes containing the higher dilutions. This is possible because the semisolid consistency of the medium permits the development of discrete colonies as shown in Fig. 2. Furthermore, the number of organisms surviving contact with an oxygenating agent can likewise be determined by counting the number of colonies developing in the subculture tubes.

In the first series of experiments an attempt was made to determine how rapidly fusiform bacilli were killed by the neutralized perborate dentifrice diluted with water, saliva, or saliva plus serum.

[1] The dentifrice (Neutrox) has the following composition: sodium perborate monohydrate (U. S. P. dehydrated), 30%; monocalcium phosphate, 30%; tricalcium phosphate, 23.87%; magnesium carbonate, 10%; saccharin soluble, 0.3%; sodium chloride, 2%; menthol, 0.33%; oil of peppermint, 0.5%; methyl salicylate, 0.65%; oil of [illegible] 0.042%; liquid petrolatum, light, 1.81%; and lauryl sulfoacetate, 0.5%. U. S. patent No. 2,014,671.

III. Hematoxylin.—Because hematoxylin also stains the basophilic granules in the mast cells and because it has proved nontoxic on oral administration (it has been used in doses as high as 5 Gm. as an astringent in diarrheas) the antiheparinic effect of this dye was tested *in vitro*. For concentrations up to 2% of heparin, it is equal in effectiveness to toluidine blue but for higher concentrations of the anticoagulant, the action of hematoxylin becomes complicated by a marked precipitation of plasma proteins.[1]

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WHO MAKES IT?

The National Registry of Rare Chemicals, Armour Research Foundation, 33rd, Federal and Dearborn Streets, Chicago, Ill., seeks information on sources of supply for the following chemicals:

Ethyl acetamidomalonate
Thioindoxyl-2-carboxylic acid
Cholinesterase
Methylphenylbarbituric acid
2,2-Dichlorocamphane
3,4-Dihydroxynorephedrine
Creatinephosphate
Stevioside
N'-3,4-Dimethylbenzoylsulfanilamide
3-Carboxy-4-hydroxypyrazole
Corynanthine
Kephalin
d-Quercitol
Chondroitin sulfuric acid
β-(2-Pyrrole)acrylic acid
Quebrachitol
Indoxyl potassium sulfate
Dipeptidase
Nucleotidase
Porphyrexide
Hydroxytyramine

The Bactericidal Action of Oxygen-Liberating Substances on Oral Microorganisms. I. Studies with Fusiform Bacilli*

By THOMAS C. GRUBB and MARGUERITE A. EDWARDS

Two freshly isolated strains of fusiform bacilli, cultured in Brewer's thioglycollate medium, were inoculated into varying concentrations of sodium perborate, hydrogen peroxide, and a neutralized perborate dentifrice, diluted with water, saliva or saliva plus serum. Subcultures were made after thirty seconds, one, two, three, five, ten, and fifteen minutes' contact. The presence or absence of growth after seven days' incubation indicated the germicidal action of the substances tested. In a dilution of 1:5 the neutralized perborate dentifrice killed all the test organisms in one minute or less. After five minutes' contact the perborate dentifrice killed the test organisms in dilutions up to 1:40 in most instances. When sodium perborate and hydrogen peroxide were compared with the perborate dentifrice in various dilutions of equal oxygen content, hydrogen peroxide was more effective and sodium perborate less effective than the perborate dentifrice in the higher dilutions. Although all three products were equally effective in the lower dilutions usually employed for oral use, the strong alkalinity of sodium perborate and the high acidity of commercial hydrogen peroxide solutions are considered undesirable properties of products intended for oral application.

ALTHOUGH oxygen-liberating substances have been used for many years in the treatment of oral infections, especially Vincent's stomatitis, there appear to be few studies published on their bactericidal properties. Indeed, with regard to sodium perborate the *United States Dispensatory* (1) states, "It is rather extraordinary that despite the tremendous quantity of it which is used today, as far as we can find there is only one experimental investigation (2) concerning its bactericidal properties which has been published." Furthermore, so far as the writers are aware, no study has been published showing the *in vitro* action of oxygen-liberating compounds on the organisms associated with Vincent's infection—*Fusiformis dentium* and *Borrelia vincenti*. Possibly the reason for this situation is that fusiform bacilli and especially oral spirochetes are relatively difficult to isolate and culture so that they may be utilized for *in vitro* tests. Since the recent literature indicates methods for isolating and culturing fusiform bacilli (3) and oral spirochetes (4, 5) it now is practicable to determine the action of oxygenating agents on these organisms. The present report describes studies on the bactericidal action of hydrogen peroxide, sodium perborate, and a neutralized sodium perborate dentifrice[1] on fusiform bacilli.

* Received June 11, 1947, from the Research Laboratories, Vick Chemical Company, Flushing, N. Y.

EXPERIMENTAL

Two strains of fusiform bacilli (G. H. and F. N.) were isolated from normal, healthy mouths of two individuals by the method of Hine and Berry (3). These strains grew readily in Brewer's thioglycollate medium (6) and corresponded morphologically (see Fig. 1) and biochemically to *Fusiformis dentium* described by Hine and Berry. The number of fusiform bacilli in a given inoculum was readily determined by making a series of successively higher dilutions in Brewer's medium, incubating the tubes for twenty-four hours at 37° and counting the number of colonies in the tubes containing the higher dilutions. This is possible because the semisolid consistency of the medium permits the development of discrete colonies as shown in Fig. 2. Furthermore, the number of organisms surviving contact with an oxygenating agent can likewise be determined by counting the number of colonies developing in the subculture tubes.

In the first series of experiments an attempt was made to determine how rapidly fusiform bacilli were killed by the neutralized perborate dentifrice diluted with water, saliva, or saliva plus serum.

[1] The dentifrice (Neutrox) has the following composition: sodium perborate monohydrate (U. S. P. dehydrated), 30%; monocalcium phosphate, 30%; tricalcium phosphate, 23.87%; magnesium carbonate, 10%; saccharin soluble, 0.3%; sodium chloride, 2%; menthol, 0.33%; oil of peppermint, 0.5%; methyl salicylate, 0.65%; oil of anise, 0.042%; liquid petrolatum, light, 1.81%; and sodium lauryl sulfoacetate, 0.5%. U. S. patent No. 2,094,671.

TABLE I.—BACTERICIDAL ACTION OF THE NEUTRALIZED PERBORATE DENTIFRICE DILUTED 1:5 ON F. DENTIUM

Contact Time	Strain G. H. Water Dil.	Strain G. H. Saliva Dil.	Strain G. H. Saliva + Serum Dil.	Strain F. N. Water Dil	Strain F. N. Saliva Dil.	Strain F. N. Saliva + Serum Dil
30 sec.	21[a]	1	0	0	2	9
1 min.	0[b]	0	0	0	0	0
2 min.	0	0	0	0	0	0
3 min.	0	0	0	0	0	0
Control	22,960	320,000	320,000	2,700,000	8,000,000	8,000,000

[a] Figures indicate number of colonies in subculture tube. [b] 0 = no growth in subculture tube.

Fig. 1.—Fusiform bacilli from blood agar culture stained with gentian violet × 750.

In performing the test 0.5 cc. of a twenty-four-hour thioglycollate culture was added to 5 cc. of the dentifrice diluted 1:5 in distilled water, sterile saliva, or saliva plus 10% sterile rabbit serum. The tubes containing the diluted dentifrice and test organisms were kept in a 37° water bath and a 4-mm. loop was used to transfer a loopful of the mixture to tubes containing 20 cc. of thioglycollate medium after thirty seconds, one, two, and three minutes' contact. The subculture tubes were incubated at 37° for seven days before recording the absence of growth, number of colonies developing, or simply the presence of growth where the number of colonies was too numerous to count.

To determine whether absence of growth in the subculture tubes indicated a bactericidal or bacteriostatic action of the dentifrice, the following test was carried out a number of times. A loopful of a 1:5 dilution of the dentifrice was inoculated into a tube of thioglycollate medium followed by a loopful of the fusiform bacilli (diluted 1:11 to correspond with the concentration used in the actual test). When these tubes were incubated growth always developed promptly and profusely indicating that the "carry over" of the dentifrice into the subculture tube was insufficient to produce bacteriostatic conditions; or, that the sodium thioglycollate in the medium inactivated any sodium perborate that might be carried over by the loop. Hence it is believed that all of the tests reported in this paper where no growth was found in the subculture tubes may be construed as indicating that the fusiform bacilli were actually killed rather than merely inhibited from growing. An additional control test consisting of fusiform bacilli suspended in water up to five minutes at 37° showed that this treatment did not reduce the number of organisms.

In this first series of tests a 1:5 dilution of the dentifrice was chosen for testing because it has been estimated that this is the approximate concentration of the dentifrice in the mouth under the usual conditions of use.

The results shown in Table I indicate the extremely rapid bactericidal action of the neutralized perborate dentifrice. It should be noted that while the dentifrice killed some 320,000 G. H. organisms and 8,000,000 F. N. organisms, it should not be concluded that the dentifrice is less effective against the G. H. strain, for these figures simply indicate the number of organisms in the inoculum and quite possibly larger numbers of G. H. organisms would have been killed if they had been present in the inoculum. Inocula containing larger numbers of the G. H. strain were not tested. While the two fusiform strains appeared to be similar morphologically and biochemically, the F. N. strain usually grew much more luxuriantly than the G. H. strain so that higher concentrations of F. N. organisms were used as the inoculum in most tests. This may explain

Fig. 2.—Fusiform bacilli growing in tubes of thioglycollate media. Note the discrete, stalactite-shaped colonies.

TABLE II.—BACTERICIDAL ACTION OF THE NEUTRALIZED PERBORATE DENTIFRICE DILUTED 1:10 TO 1:160 ON F. DENTIUM

Dilution	Water Dil. 5′	10′	15′	Saliva Dil. 5′	10′	15′	Saliva + Serum Dil. 5′	10′	15′
				Strain G. H.					
1:10	—[a]	—	—	—	—	—	—	—	—
1:20	—	—	—	—	—	—	—	—	—
1:40	—	—	—	28	—	—	+	—	—
1:80	85[b]	—	—	+	+	+	+	+	+
1:160	+[c]	+	20	+	+	+	+	+	+
				Strain F. N.					
1:10	—	—	—	—	—	—	5	—	—
1:20	—	—	—	3	—	—	5	—	—
1:40	—	—	—	+	—	—	+	1	—
1:80	+	—	—	+	+	+	+	+	+
1:160	+	+	—	+	+	+	+	+	+

[a] — = no growth in subculture tube.
[b] Figures indicate number of colonies in subculture tube.
[c] + = Colonies too numerous to count.

why the F. N. strain often showed larger numbers of survivors than the G. H. strain in the subculture tubes, although the possibility that the F. N. organisms are more resistant *per se* than the G. H. organisms is not excluded.

The presence of saliva or saliva plus serum in the dilution mixture did not appear to have much effect on the bactericidal action of the dentifrice in this series of experiments.

Since the neutralized perborate dentifrice was so effective in low dilutions it was thought of interest to determine its action in higher dilutions and longer periods of contact with the test organisms.

Here again the neutralized perborate dentifrice shows a clear-cut bactericidal action in dilutions up to 1:40 in most instances. The difference between the results obtained with the two strains of fusiform bacilli is very marked in this series of tests. The F. N. strain was not killed so readily as the G. H. strain either because of the higher concentration of organisms in the inoculum or its inherent resistance, as noted above. The presence of saliva and serum caused some reduction in bactericidal activity.

Since sodium perborate or hydrogen peroxide is often used in dental practice to treat oral infections, it seemed of interest to compare their *in vitro* bactericidal action on fusiform bacilli with that of the neutralized perborate dentifrice. The test method was the same as that employed in the preceding experiments. In preparing the dilutions of the substances to be tested, the concentration of available oxygen per Gm. or cc. was first determined and then dilutions in water were made so that the corresponding dilution of each substance contained the same amount of available oxygen; e.g., a 1:50 dilution of sodium perborate and a 1:15 dilution of the neutralized perborate dentifrice contained equivalent amounts of available oxygen. The bactericidal tests were carried out within a few minutes after preparing the dilutions of each compound.

The results shown in Table III suggest that the neutralized perborate dentifrice is somewhat more

TABLE III.—COMPARISON OF BACTERICIDAL ACTION OF THE NEUTRALIZED PERBORATE DENTIFRICE AND SODIUM PERBORATE MONOHYDRATE ON F. DENTIUM

Perborate Dentifrice Dilutions	Perborate Dentifrice: Strain G. H. 5′	10′	15′	Strain F. N. 5′	10′	15′	Sodium Perborate Dilutions	Sodium Perborate: Strain G. H. 5′	10′	15′	Strain F. N. 5′	10′	15′
1:15	—[a]	—	—	—	—	—	1:50	—	—	—	—	—	—
1:30	—	—	—	—	—	—	1:100	—	—	—	—	—	—
1:60	—	—	—	—	—	—	1:200	11	—	—	4	—	—
1:120	7[b]	—	—	—	—	—	1:400	33	—	—	30	—	—
1:240	4	—	—	—	—	—	1:800	100	—	—	5	—	—

[a] — = no growth in subculture tube.
[b] Figures indicate number of colonies in subculture tube.

TABLE IV.—COMPARISON OF BACTERICIDAL ACTION OF THE NEUTRALIZED PERBORATE DENTIFRICE AND HYDROGEN PEROXIDE ON F. DENTIUM

Perborate Dentifrice Dilutions	Perborate Dentifrice: Strain G. H. 5′	10′	15′	Strain F. N. 5′	10′	15′	Hydrogen Peroxide Dilutions	Hydrogen Peroxide: Strain G. H. 5′	10′	15′	Strain F. N. 5′	10′	15′
1:10	—[a]	—	—	—	—	—	1:2.34	—	—	—	—	—	—
1:20	—	—	—	—	—	—	1:4.68	—	—	—	—	—	—
1:40	—	—	—	—	—	—	1:9.36	—	—	—	—	—	—
1:80	1[b]	—	—	—	—	—	1:18.72	—	—	—	—	—	—
1:160	6	—	—	4	3	—	1:37.44	—	—	—	—	—	—

[a] — = no growth in subculture tube.
[b] Figures indicate number of colonies in subculture tube.

effective than sodium perborate monohydrate when compared in the higher dilutions of equal available oxygen concentration.

When the neutralized perborate dentifrice is compared with hydrogen peroxide (U. S. P.), the latter appears more effective in the higher dilutions. It is possible that the greater activity of hydrogen peroxide may result from its low *p*H (3.7 to 4.9 in the dilutions tested), while the neutralized perborate dentifrice is essentially neutral, as demonstrated by Manchey and Lee (7).

In comparing the bactericidal action of hydrogen peroxide, sodium perborate, and the neutralized perborate dentifrice it is questionable whether the classical method of comparing germicides by determining the highest dilution which fails to show growth in the subculture tubes gives a fair indication of their relative value in actual usage. It is quite possible that all three substances are equally germicidal in the low dilutions in which they are usually employed in the mouth.

DISCUSSION

Although the results reported here are based on the use of but two strains of fusiform bacilli, it is believed that the bactericidal action of the oxygen liberating compounds was so consistent and clear cut that only slight variations in the results would be anticipated with a larger number of strains.

The dilution of the oxygenating agents with saliva and serum was, of course, intended to simulate *in vivo* conditions, and although there is some indication that these substances decrease the bactericidal activity in the higher dilutions tested, there is no significant reduction in activity in the dilutions ordinarily employed in oral application.

While it was pointed out above that in the dilutions ordinarily used in the mouth, hydrogen peroxide, sodium perborate, and the neutralized perborate dentifrice may have equal bactericidal action, other factors must be taken into consideration in evaluating the usefulness of any substance designed for oral application. Other factors being equal, it would appear desirable to employ a compound which was neither strongly alkaline, as is recognized in the case of sodium perborate (*p*H 9.9 in the dilutions tested), nor quite acidic as in the case of hydrogen peroxide, due to the oxygen preservative added to commercial preparations.

The wide usage of oxygen-liberating substances in the treatment of Vincent's infection appears to be based almost entirely on the satisfactory therapeutic results obtained in most cases. A recent study conducted in an Army hospital by Wihr (8) where cases of Vincent's infection were treated with penicillin by topical application or injection and the auxiliary use of an oxygen-liberating compound as a mouth wash, seems to indicate the continued use of oxygenating compounds in modern oral therapy. The *in vitro* demonstration of the rapid bactericidal action of oxygen-liberating substances on fusiform bacilli would appear to lend experimental support to the more or less empirical usage of these substances for the treatment of oral infections.

SUMMARY

Controlled bactericidal tests with sodium perborate, hydrogen peroxide, and a neutralized perborate dentifrice diluted with water, saliva, or saliva plus serum demonstrated the ability of these substances to kill oral fusiform bacilli rapidly and with approximately equal facility in the dilutions and simulated conditions of actual usage. The choice of these oxygen-liberating agents for oral application is discussed.

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Chemical Investigation of *Premna Integrifolia* Linn.*

By N. K. BASU and P. C. DANDIYA

Two alkaloids, Premnine and Ganiarine, have been isolated from *Premna integrifolia* and their physical and chemical properties described.

Premna integrifolia Linn. or *Premna spinosa* Roxb. is a small tree or a big shrub belonging to the natural order Verbenaceae. It grows in India near the sea from Bombay to Malacca and Sylhet. It also grows in Ceylon. The common Indian names of the plant are Ganikarika (Sanskrit), Ganiari and Arni (Hindi, Bengali, and Gujrati), Munnay (Tamil).

The name "Ganiari" is very indiscriminately applied to another plant, *Clerodendron phlomoides* Roxb. belonging to the same natural order, but the latter is distinguished from the former by its vernacular synonym "Chhoti Arni" as the corresponding synonym for *P. integrifolia* is "Bari Arni."

The drug is very extensively used in the Ayurvedic system of medicine and the roots are considered to be laxative, stomachic and alexipharmic. The decoction of the roots is given in gonorrhea and during convalescence from fever. It is an important ingredient of "dasamula."

Regarding the chemical constituents of the drug, Dymock, Wardon, and Hooper (1) report the presence of an amorphous alkaloid, a substance reducing Fehling's solution, and an astringent body giving a green color with ferric chloride but giving no precipitate with gelatin. Beyond this, little work on the chemical constituents of the drug seems to have been done.

The present authors have been able to isolate two different alkaloids from the stem bark of the tree; one, Premnine, (empirical formula $C_{14}H_{15}NO$ and m. p. 82°) in pure amorphous form, from its pure crystalline hydrochloride; and the other, Ganiarine, only in the crude form. Ganiarine is extremely soluble in water, while premnine is only moderately soluble, which fact has formed the basis of separation of these alkaloids. Some derivatives of the bases have also been prepared. Some unsaturated hydrocarbons of high molecular weight have also been detected in the drug. The pharmacological action of premnine has been studied to some extent.

EXPERIMENTAL

Preliminary investigations with different solvents soon established the fact that the drug is best extracted with 90% alcohol by soxhlation. An acid solution of the crude alkaloidal bases is precipitated by Mayer's, Dragendorff's, Wagner's, and other alkaloidal reagents. Dragendorff's reagent is found to be the most suitable for the purpose.

Ten kilograms of the coarsely powdered drug was extracted with hot rectified spirit by soxhlation. The extract, on keeping, deposited some granular sediment. The clear supernatant liquid was decanted off and the sediment was reserved for investigation (A). The decantate was concentrated to a syrupy consistency and the hot syrupy liquid was slowly poured into 5 L. of warm hydrochloric acid (1%), with constant shaking. The mixture was shaken mechanically for two hours and filtered. A portion of the filtrate (500 cc.), representing 1 Kg. of the drug, was transferred to a separatory funnel and shaken with chloroform to remove the coloring matter, each chloroform extract being washed with the same 1% HCl kept in another separator. The acid liquid was then rendered alkaline with dilute ammonia and was shaken repeatedly with chloroform. It was observed that even 2 L. of chloroform could not extract the alkaloids completely. Extraction with chloroform was continued after successive addition of stronger alkalies like sodium carbonate and sodium hydroxide, but this also did not help in the complete extraction of the alkaloids. The aqueous liquid which still gave a strong test with Dragendorff's reagent was made neutral to litmus and reserved (B).

The rest of the acid extract was extracted with chloroform by the above process and the combined chloroform extract was dried over anhydrous sodium sulfate and filtered. The filtrate on distillation left behind a dark brown residue weighing 4.2 Gm.

Purification.—The residue was dissolved in a few cubic centimeters of chloroform and gasoline was added dropwise, whereby a lot of resinous matter precipitated. The supernatant liquid was decanted off and evaporated; the residue was again dissolved in a little chloroform and ether was added to it in excess, when black resins separated. The above process was repeated several times. The

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product finally obtained was yellow in color. Efforts were made to crystallize the base from various solvents, but it did not show a tendency to crystallize.

Preparation of the Hydrochloride.—The dry, yellowish semipurified alkaloidal residue was dissolved in dry ether and a few drops of absolute alcohol saturated with HCl gas were added to it drop by drop, when a yellowish precipitate was obtained. The precipitate obtained was dissolved in absolute alcohol and crystallized. The hydrochloride as finally obtained is in the form of colorless acicular crystals melting at 211–213°. The yield obtained was 65 mg. The name Premnine hydrochloride is suggested for it by the authors. A microphotograph of the hydrochloride is shown in Fig. 1.

Figure 1

Preparation of the Base.—A portion of the hydrochloride was rubbed with moist silver oxide and a few cubic centimeters of water was added and the whole was again mixed thoroughly. The mixture was filtered and the filtrate on evaporation at 70° gave an amorphous base melting at 82°. It did not crystallize from any of the common solvents. The following derivatives were prepared: premnine picrate, m. p. 98–101° (with decomp.); premnine platinum chloride, decomposing at 254–256°.

On submitting the hydrochloride to semimicro combustion analysis, the following results were obtained: Carbon, 67.88; Hydrogen, 6.096; Nitrogen, 5.551; Chlorine, 14.41; Oxygen (by difference), 6.063.

By dividing the proportionate numbers of all the elements by the number of nitrogen, the empirical formula comes to $C_{14}H_{15}NO$. The empirical weight calculated from this formula is 213.

A weighed amount of the platinum chloride derivative was ignited in a microcrucible and the residue weighed. Assuming the base to be mono-acidic the molecular weight calculated from the data obtained is 212 and compares very closely with that obtained by calculation from the empirical formula.

Ganiarine.—To the neutral, aqueous solution "B" reserved previously was added an excess of strong solution of lead subacetate (B. P.) when a bulky precipitate of lead tannate was obtained. The precipitate was filtered off and H_2S gas was passed in the filtrate to precipitate the excess of lead as lead sulfide. After passing a current of air to remove the dissolved H_2S gas the bright yellow filtrate was evaporated to 50 cc. under reduced pressure. The concentrated extract was made alkaline with ammonia and again extracted with chloroform. It was observed that even 500 cc. of chloroform extracted very little alkaloid. The aqueous liquid was once again made neutral and evaporated to dryness *in vacuo*. The residue was extracted with absolute alcohol, which eliminated most of the salts formed during the previous processes. The alcoholic extract was concentrated and an excess of ether was added to it, when resins and more salts separated. The process was repeated several times and the product finally obtained by evaporation of the ethereal extract, was yellowish brown in color. Further purification of the alkaloid could not be attempted. The platinum chloride derivative which could be prepared from the crude base decomposes at 239–241°.

Hydrocarbons.—The granular sediment "A," reserved previously, on examination was found to be a mixture of unsaturated aromatic hydrocarbons of high molecular weight.

Pharmacology of Premnine

(a) On Blood Vessels.—The action of premnine on blood vessels was studied by determining the perfusion rate in a frog. First of all the number of drops of frog-Ringer Solution, flowing per minute was counted. Then 0.5 cc. of 1:5000 solution of premnine hydrochloride was injected into the rubber tubing connecting the reservoir of the frog-Ringer Solution to the ventricle of the frog and the number of drops per minute after the injection was counted. Three such readings were taken in each case. The mean of the readings was 37 and 27, respectively. The results show that the drug raises the blood pressure by contracting the blood vessels, i.e., a sympathomimetic drug.

Figure 2

(b) On the Heart.—Heart of a pithed frog was exposed and frequently perfused with frog-Ringer

Solution. The ventricle of the heart was connected through a bent hook with the cardiograph lever attached with a small pointer touching the smoked paper fixed to the rotating drum. The curves indicating the normal contractions of the heart and also the contractions after perfusion with 1:5000 solution were recorded (Fig. 2). As is apparent from the recorded tracings the drug decreases the force of contraction of a frog's heart.

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1. Two alkaloids—one, premnine in pure amorphous form (m. p. 82°, empirical formula $C_{14}H_{15}NO$) and the other, "ganiarine" only in crude form (platinum chloride derivative decomposes at 239–241°)—have been isolated from the stem bark of *Premna integrifolia*.

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The Estimation of Aloin in Pharmaceuticals*

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A polarographic method for determination of aloin in pharmaceuticals has been applied to seven commercial preparations.

THE USE of aloin in the form of aloes or aloin isolated from aloes for pharmaceutical purposes is very old. There has been an obvious need for an analytical method for the estimation of aloin both in raw materials and in finished products. Previous methods have usually involved either extraction of the aloin or precipitation of calcium aloinate, both of which have been criticized on many points. Recently Stone and Furman (1) have utilized the polarographic method for the analysis of aloes. It is the purpose of this paper to describe the analysis of pharmaceuticals for aloin by this method.

THE POLAROGRAPHIC METHOD

The polarographic method may be briefly described as an electrolysis under very strictly controlled conditions so that the current flowing at any moment may be accurately measured. Organic groups such as carbonyl, nitro, azo, quinone, and conjugated double bonds are reducible, and the potentials required for the reduction of these groups are sufficiently different that the potential is characteristic for the particular group. The electrolytic measurements are made by measuring the current that flows at increasing potentials with a dropping mercury electrode and a quiet pool of mercury as the other electrode with no stirring of the solution. The mercury drop is usually the cathode with reduction occurring.

If the solution being electrolyzed contains an electrolyte, preferably a buffer solution to eliminate *p*H effects, no appreciable current flows until enough potential is applied to reduce something in the solution. As the potential increases, more current will flow until all of the material approaching the mercury drop is reduced. Then the current will become constant, since more material will approach the drop at a constant rate due to diffusion. The difference in current before and after this rise which is usually called "wave height" is proportional to the concentration of material being reduced.

A more detailed treatment may be found in Kolthoff and Lingane (2).

THE ESTIMATION OF ALOIN

Stone and Furman (1) found that there were no interfering substances in aloes, so that a determination could be made. (Other substance that was reduced at the same as aloin would contribute to the and thus interfere.) Commercial were analyzed by the same that follow.

* Received March 15, 1947, from the Frick Chemical Laboratory, Princeton University, Princeton, N. J. Present address: Kedzie Chemical Lab., Michigan State College, E. Lansing, Mich.

product finally obtained was yellow in color. Efforts were made to crystallize the base from various solvents, but it did not show a tendency to crystallize.

Preparation of the Hydrochloride.—The dry, yellowish semipurified alkaloidal residue was dissolved in dry ether and a few drops of absolute alcohol saturated with HCl gas were added to it drop by drop, when a yellowish precipitate was obtained. The precipitate obtained was dissolved in absolute alcohol and crystallized. The hydrochloride as finally obtained is in the form of colorless acicular crystals melting at 211–213°. The yield obtained was 65 mg. The name Premnine hydrochloride is suggested for it by the authors. A microphotograph of the hydrochloride is shown in Fig. 1.

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Stone and Furman (1) found that there were no interfering substances in aloes, so that a direct determination could be made. (Obviously, any other substance that was reduced at the same potential as aloin would contribute to the increase in current, and thus interfere.) Commercial pharmaceuticals were analyzed by the same method with the results that follow.

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APPARATUS, MATERIALS, AND METHOD

A Leeds and Northrup Electrochemograph equipped with the cell arrangement described by Furman, Bricker, and Whitesell (3) was used for the polarographic measurements at room temperature. The capillary had the following characteristics: m = 0.8173 mg. per second, t = 7.60 seconds at −1.34 volt against the saturated calomel electrode (S. C. E.) in the buffer solution; the head of mercury was 46.5 cm. Oxygen was removed by passing purified nitrogen through the solution for fifteen minutes before polarographing. All polarograms were taken at 1/50 sensitivity.

The 0.25 M acetate buffer of pH 4 was prepared by neutralizing 0.25 mole of glacial acetic acid in 600 ml. of water with concentrated sodium hydroxide solution to pH 4 measured against a glass electrode and diluting to 1000 ml. A polarogram of the buffer alone showed no appreciable amount of reducible material before hydrogen evolution.

Merck U. S. P. aloin was used without further purification. For a standard solution, 1.000 Gm. of aloin was dissolved in 1000 ml. of 20 volume per cent ethanol. This solution was stable for about one week when protected from light and air.

Procedure.—Weigh about 1.5-Gm. sample to the nearest milligram (for aloes, 0.2 Gm. to the nearest 0.1 mg.). Warm in 20 ml. 95% ethanol and 25 ml. water for fifteen minutes just below the boiling point. Macerate with stirring rod if necessary. Cool to room temperature, filter on paper into a 100-ml. volumetric flask, wash the residue with three 10-ml. portions of distilled water collecting the wash solution with the original filtrate, and dilute to the mark. Mix a 10-ml. aliquot with 10 ml. of the acetate buffer and dilute to 25 ml. Deaerate with nitrogen, and take a polarogram from −1.0 volt applied potential. Compare the wave-height with the calibration curve.

Preparation of the calibration curve: Mix suitable volumes of the standard aloin solution with 10 ml. of the acetate buffer, add ethanol to give a total concentration of eight volume per cent ethanol in the final solution, and dilute to 25 ml. Proceed as with the sample. The calibration curve is usually a straight line for concentrations up to 15 mg. of aloin per 25 ml. of the solution polarographed. The most critical factor is the ethanol concentration, since the reducible species is quite dependent on the solvent composition. If the composition is not constant, the calibration curve will not be a straight line. It was also noticed that an old standard solution of aloin also led to deviations from a straight line calibration. Various lots of aloin seemed to have very little effect on the current-concentration ratio.

In order to check the working procedure, a series of recovery studies was made. A sample of pure barbaloin was prepared according to the procedure of Gardner and Joseph (4). This barbaloin was used as a reference standard for all recovery studies of which the following are typical:

1. A sample of aloin equal to 10.0 mg. of the pure barbaloin was analyzed and 10.2 mg. of barbaloin were found.
2. A mixture of 5.0 mg. of barbaloin and aloes equivalent to 4.2 mg. of barbaloin was found to contain 9.1 mg. of barbaloin.
3. A pharmaceutical which was said (5) to cotain 5 mg. of aloin per tablet was analyzed and 4.7 mg. per tablet were found.

RESULTS

In Table I will be found the per cent of aloin found in each of seven preparations. These products are from seven different manufacturers, and consist of various mixtures of aloes or aloin, soap, assorted gums, podophyllin, extracts of belladonna, stramonium, etc., ipecac, phenolphthalein, and flavors. It is not possible to tell the amount of aloes present in the preparations containing aloes unless the relation between aloin and aloes is known since the amount of aloin in aloes varies from lot to lot depending on the source. Hence, in order to compare the preparations, all of which were recommended for treatment of colds, the assay was combined with the recommended dose on the label to give the recommended dose in terms of aloin. It is interesting to note that the dose as aloin varies 25% from the average.

TABLE I.—DETERMINATION OF ALOIN IN PHARMACEUTICALS

Preparation	% Aloin	Dose, Gm. Aloin
A	6.1	0.0138
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C	7.9	0.0225
D	9.5	0.0355
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F	18.1	0.0172
G	24.6	0.0207
	Av.	0.0237

Very few materials are known that are reduced in this potential range that also occur in the pharmaceutical raw materials used. Hence an interference in this determination is possible, but not probable. The usual polarographic analysis has an inherent error of about three relative per cent so that error may be anticipated in this determination.

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Pharmacognostical Studies of Brahmi

Stem and Leaf Characteristics of *Herpestis monniera* H. B. and K. and *Hydrocotyle asiatica* Linn.*

By S. PRASAD†

This report includes new information on the pharmacognosy of the drug known in India as Brahmi. The results reported will serve to distinguish between the two plant species involved in connection with Brahmi.

A VERY important drug in the Ayurvedic Materia Medica of India, Brahmi has been used by the Hindu physician as a nervine tonic from very ancient times. It has been considered useful also in epilepsy and insanity and has been presented to the Western physician, practicing in India, in the form of syrup, liquid extract, ghee, and hair oil by many pharmaceutical manufacturing houses of that country.

The correct identity of this reputed drug has, however, not yet been ascertained. While one group of Ayurvedic physicians regards *Herpestis monniera* H. B. and K. (Fam. Scrophulariaceae) to be Brahmi, there are others who consider *Hydrocotyle asiatica* Linn. (Fam Umbelliferae) as the real drug. Recently Sircar (1) tried to establish, on the basis of botanical characters embodied in the Ayurved that *Hydrocotyle asiatica* and not *Herpestis monniera* is the real Brahmi. In the drug market both these species are sold indiscriminately as Brahmi.

A fair amount of chemical and pharmacological work has already been done on these two plant species (2–4). No pharmacognostical study on any of these plant species has yet been brought on record. The present investigation was, therefore, taken up with a view to bring out all those diagnostic features which may help one to distinguish one species from the other. The anatomy of the leaf and of the stem, along with their macroscopical characteristics, has been studied in detail in both the species. The investigation has been extended to the determination of their stomatal indices and palisade ratios as well, to ascertain if these values could be utilized for distinguishing the two species.

* Received June 24, 1947.
† Of the Department of Pharmaceutics, Benares Hindu University, Benares, India; now at the School of Pharmacy, University of Wisconsin, Madison, Wis.

MATERIALS AND METHODS

Fresh specimens of *Herpestis monniera* and *Hydrocotyle asiatica* were collected from the Ayurvedic garden of the Benares Hindu University. Free hand sections from the different portions of stem, leaf, and petiole were stained in safranin and gentian violet and mounted in Canada balsam in the usual way. Surface peelings were examined in chloral hydrate solution. The palisade ratio and stomatal index were determined by the following procedure:

Pieces of leaf tissue, 1–3 mm. square, were cut from the base, apex, and the middle portions of the leaf and placed in test tubes containing concentrated chloral hydrate solution (50 Gm. chloral hydrate dissolved in 20 cc. water). These were then placed in a water bath for about twenty minutes or more until they became more or less transparent. The palisade ratio was then determined according to the method employed by Wallis and Dewar (5).

For determining stomatal index, similar procedure was adopted so far as the clearing of leaves was concerned. The stomatal indices were found as proposed by Salisbury (6), that is, $I = S/(E + S) \times 100$, where S is the number of stomata per unit area, and E the number of epidermal cells in the same unit area. During the process of determination of stomatal values, two guard cells were considered as one unit, each hair one unit, and each epidermal cell one unit. To determine if the stomatal index and palisade ratio varied significantly from locality to locality, the data pertaining to these were statistically analyzed to test the significance of the result.

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External Morphology

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APPARATUS, MATERIALS, AND METHOD

A Leeds and Northrup Electrochemograph equipped with the cell arrangement described by Furman, Bricker, and Whitesell (3) was used for the polarographic measurements at room temperature. The capillary had the following characteristics: m = 0.8173 mg. per second, t = 7.60 seconds at −1.34 volt against the saturated calomel electrode (S. C. E.) in the buffer solution; the head of mercury was 46.5 cm. Oxygen was removed by passing purified nitrogen through the solution for fifteen minutes before polarographing. All polarograms were taken at 1/50 sensitivity.

The 0.25 M acetate buffer of pH 4 was prepared by neutralizing 0.25 mole of glacial acetic acid in 600 ml. of water with concentrated sodium hydroxide solution to pH 4 measured against a glass electrode and diluting to 1000 ml. A polarogram of the buffer alone showed no appreciable amount of reducible material before hydrogen evolution.

Merck U. S. P. aloin was used without further purification. For a standard solution, 1.000 Gm. of aloin was dissolved in 1000 ml. of 20 volume per cent ethanol. This solution was stable for about one week when protected from light and air.

Procedure.—Weigh about 1.5-Gm. sample to the nearest milligram (for aloes, 0.2 Gm. to the nearest 0.1 mg.). Warm in 20 ml. 95% ethanol and 25 ml. water for fifteen minutes just below the boiling point. Macerate with stirring rod if necessary. Cool to room temperature, filter on paper into a 100-ml. volumetric flask, wash the residue with three 10-ml. portions of distilled water collecting the wash solution with the original filtrate, and dilute to the mark. Mix a 10-ml. aliquot with 10 ml. of the acetate buffer and dilute to 25 ml. Deaerate with nitrogen, and take a polarogram from −1.0 volt applied potential. Compare the wave-height with the calibration curve.

Preparation of the calibration curve: Mix suitable volumes of the standard aloin solution with 10 ml. of the acetate buffer, add ethanol to give a total concentration of eight volume per cent ethanol in the final solution, and dilute to 25 ml. Proceed as with the sample. The calibration curve is usually a straight line for concentrations up to 15 mg. of aloin per 25 ml. of the solution polarographed. The most critical factor is the ethanol concentration, since the reducible species is quite dependent on the solvent composition. If the composition is not constant, the calibration curve will not be a straight line. It was also noticed that an old standard solution of aloin also led to deviations from a straight line calibration. Various lots of aloin seemed to have very little effect on the current-concentration ratio.

In order to check the working procedure, a series of recovery studies was made. A sample of pure barbaloin was prepared according to the procedure of Gardner and Joseph (4). This barbaloin was used as a reference standard for all recovery studies of which the following are typical:

1. A sample of aloin equal to 10.0 mg. of the pure barbaloin was analyzed and 10.2 mg. of barbaloin were found.
2. A mixture of 5.0 mg. of barbaloin and aloes equivalent to 4.2 mg. of barbaloin was found to contain 9.1 mg. of barbaloin.
3. A pharmaceutical which was said (5) to contain 5 mg. of aloin per tablet was analyzed and 4.7 mg. per tablet were found.

RESULTS

In Table I will be found the per cent of aloin found in each of seven preparations. These products are from seven different manufacturers, and consist of various mixtures of aloes or aloin, soap, assorted gums, podophyllin, extracts of belladonna, stramonium, etc., ipecac, phenolphthalein, and flavors. It is not possible to tell the amount of aloes present in the preparations containing aloes unless the relation between aloin and aloes is known since the amount of aloin in aloes varies from lot to lot depending on the source. Hence, in order to compare the preparations, all of which were recommended for treatment of colds, the assay was combined with the recommended dose on the label to give the recommended dose in terms of aloin. It is interesting to note that the dose as aloin varies 25% from the average.

TABLE I.—DETERMINATION OF ALOIN IN PHARMACEUTICALS

Preparation	% Aloin	Dose, Gm. Aloin
A	6.1	0.0138
B	7.5	0.0224
C	7.9	0.0225
D	9.5	0.0355
E	13.8	0.0335
F	18.1	0.0172
G	24.6	0.0207
	Av.	0.0237

Very few materials are known that are reduced in this potential range that also occur in the pharmaceutical raw materials used. Hence an interference in this determination is possible, but not probable. The usual polarographic analysis has an inherent error of about three relative per cent so that error may be anticipated in this determination.

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Pharmacognostical Studies of Brahmi

Stem and Leaf Characteristics of *Herpestis monniera* H. B. and K. and *Hydrocotyle asiatica* Linn.*

By S. PRASAD†

This report includes new information on the pharmacognosy of the drug known in India as Brahmi. The results reported will serve to distinguish between the two plant species involved in connection with Brahmi.

A VERY important drug in the Ayurvedic Materia Medica of India. Brahmi has been used by the Hindu physician as a nervine tonic from very ancient times. It has been considered useful also in epilepsy and insanity and has been presented to the Western physician, practicing in India, in the form of syrup, liquid extract, ghee, and hair oil by many pharmaceutical manufacturing houses of that country.

The correct identity of this reputed drug has, however, not yet been ascertained. While one group of Ayurvedic physicians regards *Herpestis monniera* H. B. and K. (Fam. Scrophulariaceae) to be Brahmi, there are others who consider *Hydrocotyle asiatica* Linn. (Fam Umbelliferae) as the real drug. Recently Sircar (1) tried to establish, on the basis of botanical characters embodied in the Ayurved that *Hydrocotyle asiatica* and not *Herpestis monniera* is the real Brahmi. In the drug market both these species are sold indiscriminately as Brahmi.

A fair amount of chemical and pharmacological work has already been done on these two plant species (2–4). No pharmacognostical study on any of these plant species has yet been brought on record. The present investigation was, therefore, taken up with a view to bring out all those diagnostic features which may help one to distinguish one species from the other. The anatomy of the leaf and of the stem, along with their macroscopical characteristics, has been studied in detail in both the species. The investigation has been extended to the determination of their stomatal indices and palisade ratios as well, to ascertain if these values could be utilized for distinguishing the two species.

* Received June 24, 1947.

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MATERIALS AND METHODS

Fresh specimens of *Herpestis monniera* and *Hydrocotyle asiatica* were collected from the Ayurvedic garden of the Benares Hindu University. Free hand sections from the different portions of stem, leaf, and petiole were stained in safranin and gentian violet and mounted in Canada balsam in the usual way. Surface peelings were examined in chloral hydrate solution. The palisade ratio and stomatal index were determined by the following procedure:

Pieces of leaf tissue, 1–3 mm. square, were cut from the base, apex, and the middle portions of the leaf and placed in test tubes containing concentrated chloral hydrate solution (50 Gm. chloral hydrate dissolved in 20 cc. water). These were then placed in a water bath for about twenty minutes or more until they became more or less transparent. The palisade ratio was then determined according to the method employed by Wallis and Dewar (5).

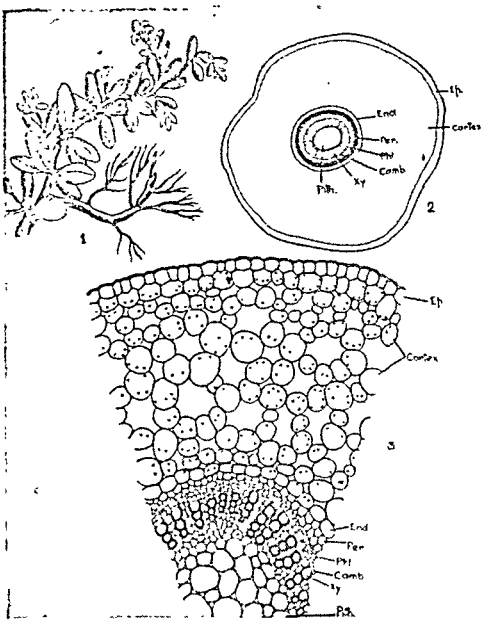
For determining stomatal index, similar procedure was adopted so far as the clearing of leaves was concerned. The stomatal indices were found as proposed by Salisbury (6), that is, $I = S/(E + S) \times 100$, where S is the number of stomata per unit area, and E the number of epidermal cells in the same unit area. During the process of determination of stomatal values, two guard cells were considered as one unit, each hair one unit, and each epidermal cell one unit. To determine if the stomatal index and palisade ratio varied significantly from locality to locality, the data pertaining to these were statistically analyzed to test the significance of the result.

INVESTIGATION

External Morphology

Herpestis monniera.—It is a creeping, somewhat succulent herb, found in moist places near streams or on the banks of ponds and ditches. It grows throughout the plains of India, ascending to 4000-ft. altitude on the Himalayas and in all warm countries.

Several stems, 4–12 inches long, arise from the main stem. The plant roots at the nodes. The branches are ascending. The leaves are sessile, decussate, 1/4–1 inch long, 0.20–0.35 inch broad,



Herpestis monniera H. B. K.

Fig. 1.—The whole plant with flowers. × 1/3.
Fig. 2.—Plan of the transverse section of the stem. × 20.
Fig. 3.—Details of transverse section. × 65.
Ep., epidermis; End., endodermis; Per., pericycle; Phl., phloem; Camb., cambium; Xy., xylem.

obovate-oblong or spatulate, entire, nerves obscure and lower surface dotted (Fig. 1). Flowers are axillary and solitary, borne on slender pedicels 1/4–1 1/4 inches long. Bracteoles are linear, shorter than the pedicels. Calyx is divided to the base, glabrous, upper segments ovate acute, longer and broader than the other. Corola is 1/3 inch long, blue or white with purple veins; lobes are nearly equal and rounded, glistening with shining dots when fresh. Capsules are 1/5 inch long, ovoid, acute, and glabrous.

Hydrocotyle asiatica.—It is an annual herb, creeping along the ground and rooting at the nodes (Fig. 11). It grows throughout India, Ceylon, and Malaya and in all the tropical and subtropical regions of the world.

Leaves, in the Indian species, are cordate or hastate, orbicular or reniform, crenate and often palmately lobed. They are 1/2–2 1/2 inches each way, glabrous or nearly so and shining, with stipules adnate to the petioles. Peduncles are much shorter than the leaves, often 2–3-nate, pubescent. Bracts are small, ovate, embracing the flowers and not scattered among the pedicels (Fig. 11 (*A*)). Flowers are sessile and grouped 3–6 in each head. Petals are minute, ovate, acute, and slightly imbricate; carpels oblong, subcylindrical, curved, much longer than broad, slightly compressed, reticulate-rugose, each with 9 curvilinear, subsimilar ridges and 2 within the commissure. The fruit is 1/8–1/6 inch in diameter (Fig. 11 (*B*)). Pericarp is thickened, woody, and white (Fig. 11 (*C*)).

Microscopical Features

Herpestis monniera

Stem.—The transverse section of the stem indicates that it consists of a single layer of epidermis, followed by a wide parenchymatous cortex and a ring of stele which encloses in the center a small amount of pith (Fig. 2). The epidermis is covered externally by a thin layer of cuticle. The epidermal cells are small and cubical and measure 20–30–38 microns × 24–34–40 microns.[1]

Beneath the epidermis is a wide zone of cortex which is composed of thin-walled, isodiametric and almost round cells with very large intercellular spaces that serve as air chambers (Fig. 3). The cortex is thus lacunar. The cortical cells measure 30–51–85 microns in diameter. The bigger cells occupy the middle region of the cortex and are in some cases tangentially elongated. The innermost layer of the cortex is represented by a distinct endodermis. The endodermal cells show Casparian strips in cross section and measure 28–35–45 microns × 18–30–35 microns.

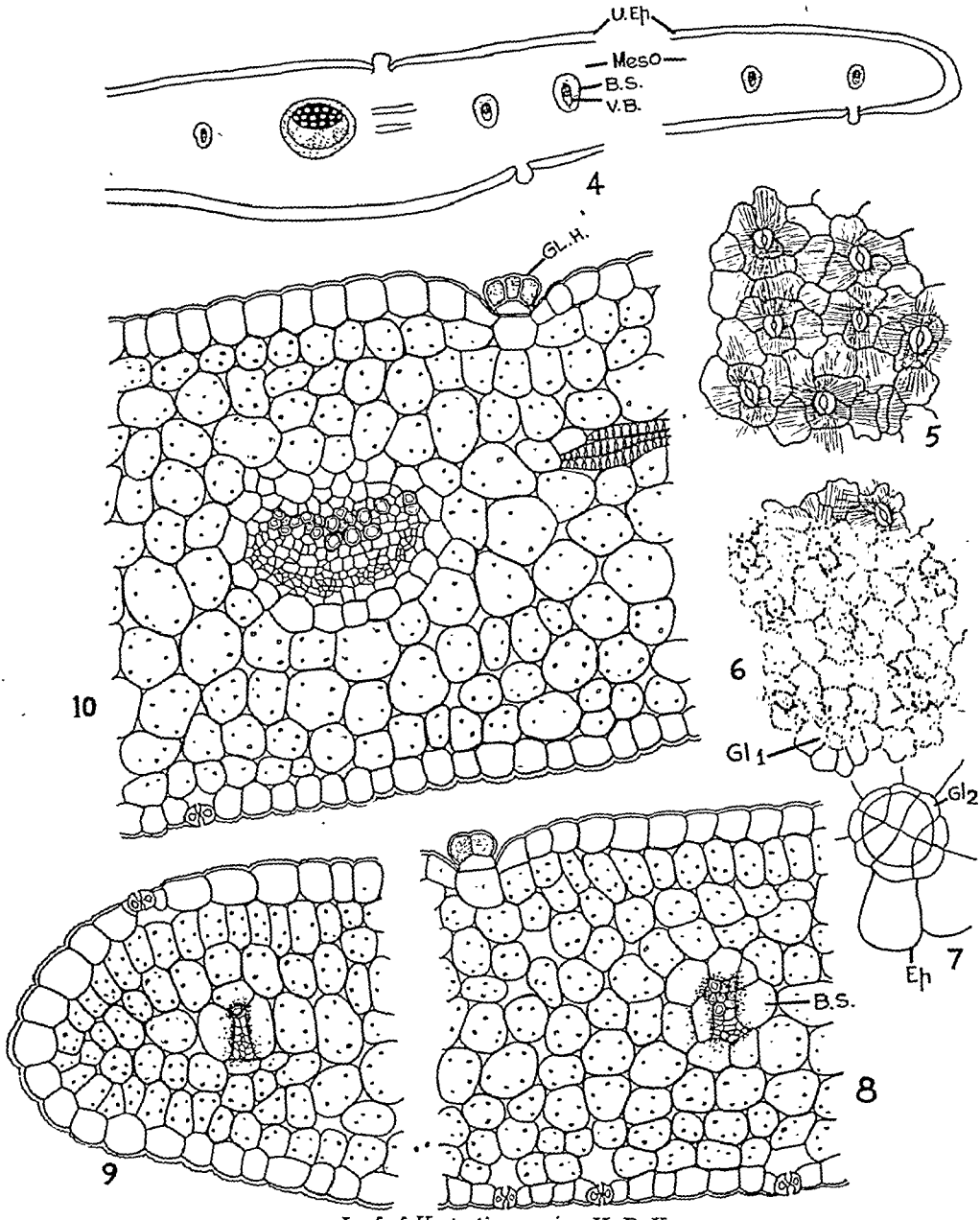
Following the endodermis is a thin strip of pericycle, consisting of 1–2 layers of cells (Fig. 3). The pericycle surrounds the stele all around and the pericyclic cells are very small, measuring 12–15–20 microns in length and 9–12–17 microns in breadth.

The vascular ring is continuous, being composed of a narrow zone of phloem toward the peripheral side, a wide ring of xylem toward the center, and a layer of cambium separating the two. The phloem consists of sieve tube tissue associated with companion cells and phloem parenchyma. The xylem is composed of xylem vessels with intervening xylem parenchyma. The vessels are polygonal and isodiametric and arranged in radial rows. They are 14–22–30 microns in diameter in transverse section. The xylem parenchyma consists of thin-walled cells, measuring 8–13–17 microns in length and 7–11–14 microns in breadth.

In the center is a small pith, the cells of which are thin walled, rounded, more or less isodiametric with distinct intercellular spaces. They are 28–38–55 microns in diameter.

Leaf.—The structure of the leaf of *Herpestis monniera* resembles more an isobilateral type of leaf than a dorsiventral one, in that it shows a lack of differentiation of the mesophyll in the palisade and spongy layers (Figs. 8–10). The surface of the leaf is covered externally with cuticle which occurs in the form of striations. These striations are more strongly developed on the lower surface than on the upper one. In surface view, the arrangement of the cuticular striations is such that they appear to

[1] The measurements of cells and other structures are given ordinarily in three separate figures, of which the first and the third represent the minimum and the maximum values, respectively, while the middle indicates the common value.



Leaf of *Herpestis monniera* H. B. K.

Fig. 4.—Transverse section of the leaf. × 45.

Fig. 5.—Upper epidermis in surface view. × 180.

Fig. 6.—Lower epidermis in surface view. × 180.

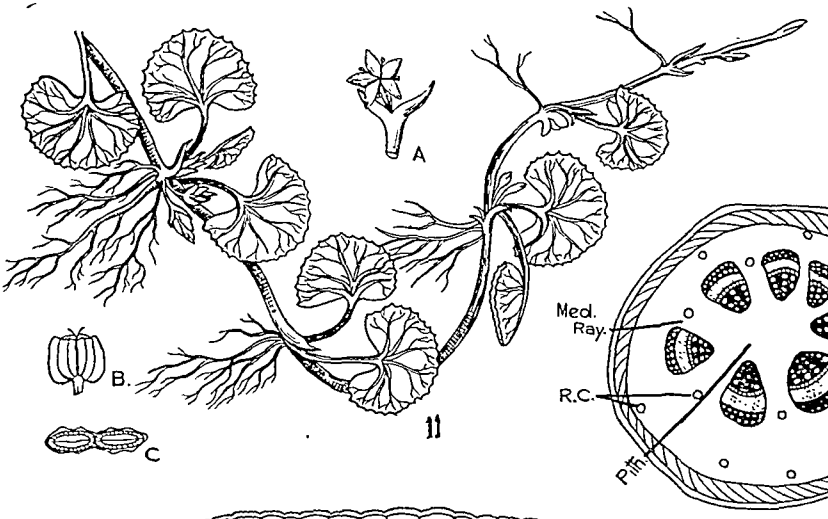
Fig. 7.—Glandular hair in surface view. × 450.

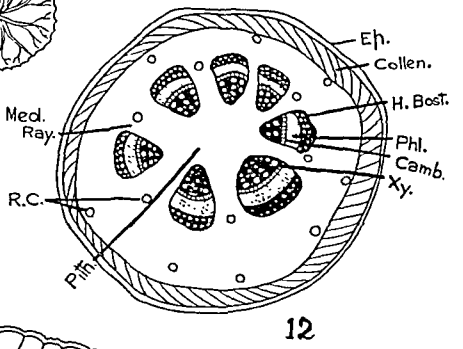
Fig. 8.—Details of transverse section of the leaf. × 225.

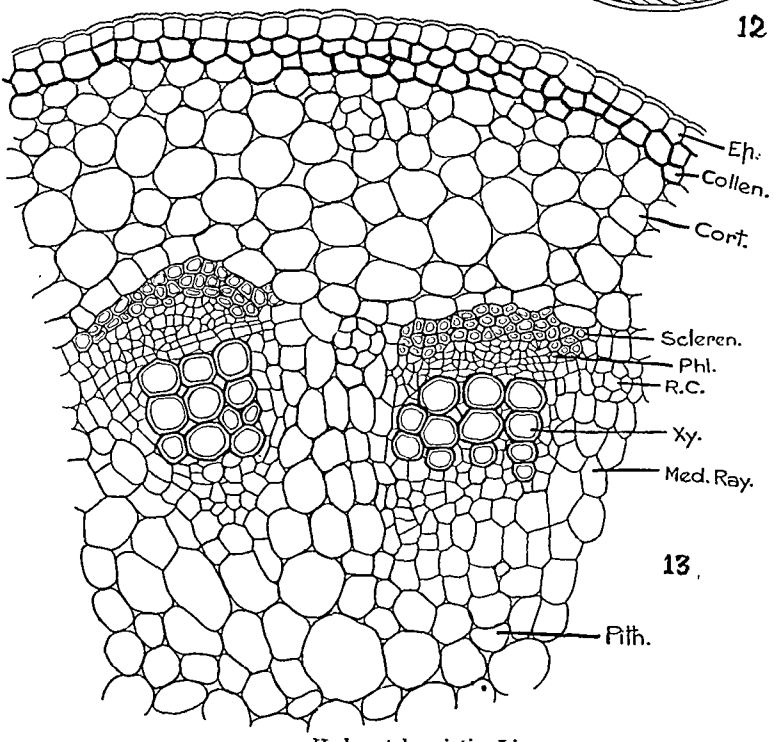
Fig. 9.—Transverse section of the margin of the leaf. × 225.

Fig. 10.—Transverse section passing through the midrib. × 225.

U.Ep., upper epidermis; Meso, mesophyll; B.S., bundle sheath; V.B., vascular bundle; Gl.H., glandular hair; Gl_1, glandular hair in surface view, showing quadrucellular head; Gl_2, the same with a head of 8 cells in surface view.







Hydrocotyle asiatica Linn.

Fig. 11.—A part of the plant. × 3/4. *A*, inflorescence. × 2 1/2. *B*, fruit. × 3. *C*, fruit cut across. × 3.

Fig. 12.—Transverse section of the stem. × 40.

Fig. 13.—Details of transverse section. × 225.

Ep., epidermis; Collen., collenchyma; Cort., cortex; Scleren., sclerenchyma; Phl., phloem; R.C., resin canal; Xy., xylem; Med. Ray, medullary ray; H. Bast, hard bast; Camb., cambium.

be diverging away from stomata in all directions (Figs. 5 and 6). In transverse section, the striations are to be seen as small ridges which are more pronounced toward the margins of the leaves (Fig. 9).

Stomata are found on both the surfaces of the leaf. There are no subsidiary cells surrounding the stomata. According to Vesque (7) their development follows the cruciferous type, but this is frequently obscured in the mature leaf; that is to say, the stomata are surrounded by three or more epidermal cells. In surface view the stomata of the upper surface (Fig. 5) measure 25–28–30 microns in length and 18–20–23 microns in breadth with the aperture being 4–6 microns wide. The stomata on the lower surface (Fig. 6) are more or less of similar dimensions as noted in the case of the upper ones.

Epidermal cells, in surface view, are much more wavy walled on the lower surface than on the upper one (Figs. 5 and 6). They measure 34–51–64 microns × 20–30–38 microns on the upper surface and 38–50–68 × 22–28–34 microns on the lower side.

Glandular hairs are present on both the surfaces. They occur in slight depressions of the lamina (Figs. 8 and 10). Some of them are smaller than others. The smaller ones are round or oval and quadricellular, borne upon a slightly conical stalk, while the larger ones possess the glandular head of eight cells. The epidermal cell joining the stalk bulges out and helps in the elongation of the stalk. In surface view the hairs are seen as circles divided into four or eight cells (Figs. 6 and 7). The glandular heads have a diameter of 28–35–44 microns. The distribution of these hairs, stomata, and epidermal cells per sq. mm. leaf surface (Table I) indicates that the upper surface of the leaf has comparatively more hairs and less stomata than the lower one.

TABLE I.—NUMBER OF STOMATA, HAIRS, AND EPIDERMAL CELLS PER SQ. MM. LEAF SURFACE IN *Herpestis monniera*

Surface	Stomata	Hairs	Epidermal Cells
Upper	65–95–128	20–32	473–615–717
Lower	90–115–141	13–26	485–620–757

In transverse section the epidermal cells are somewhat vertically elongated, measuring 14–24–35 microns × 30–35–42 microns in case of the upper surface and 15–30–35 microns × 20–25–30 microns on the lower surface. The palisade tissue is weakly developed, the mesophyll cells being 35–45–60 microns in length and 22–30–40 microns in breadth. The spongy mesophyll, in the center, is rather more compactly arranged and the cells are bigger and more or less isodiametric with a diameter of 30–56–80 microns. Toward the dorsal side, the spongy layer is somewhat loosely arranged and the cells are smaller in dimensions. This meager difference between the palisade and spongy cells is still less marked in the margin region (Fig. 9). A few prismatic crystals of calcium oxalate are occasionally found distributed in the mesophyll cells.

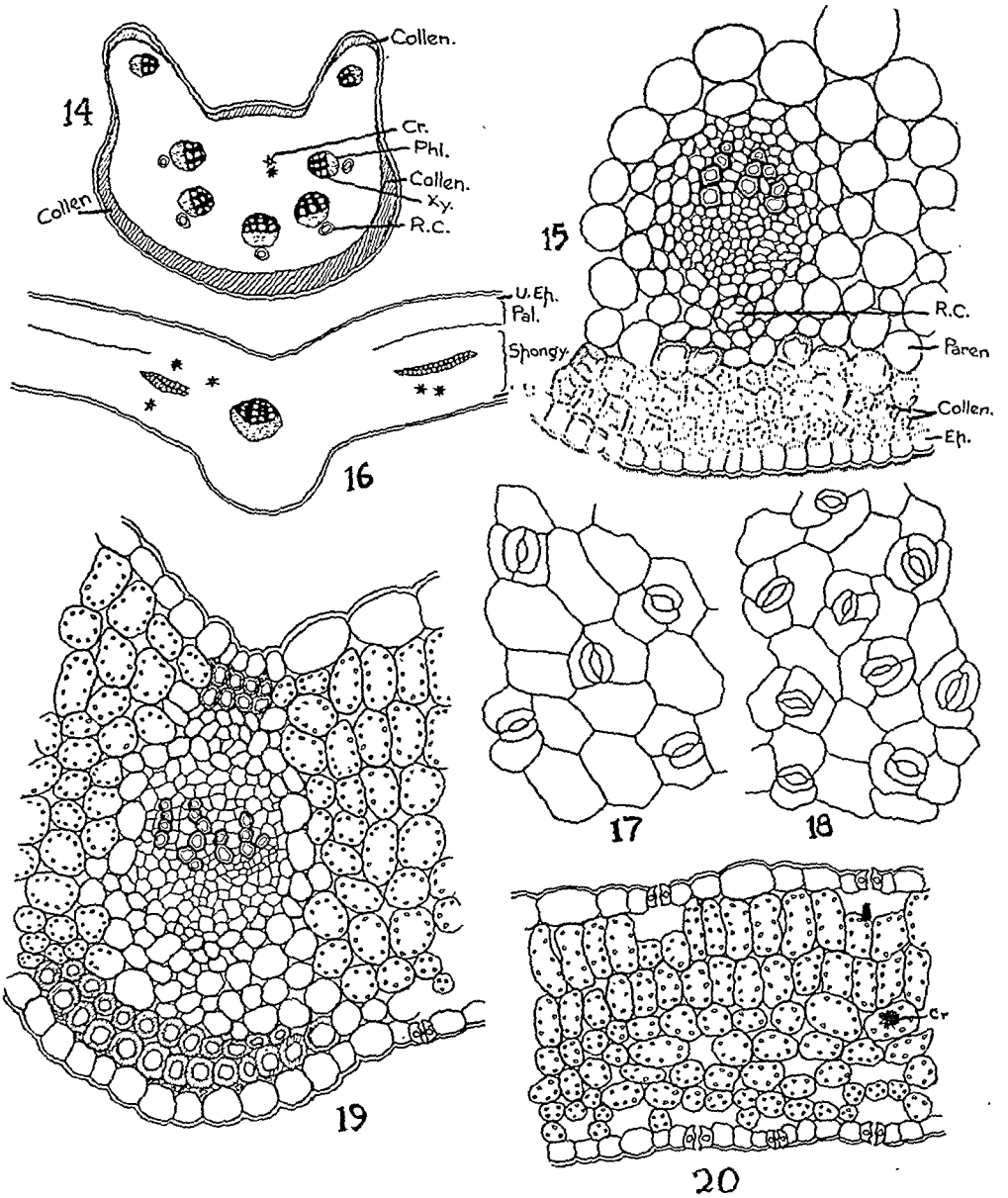
The mesophyll is traversed by a number of small veins which are surrounded by bundle sheaths (Figs. 8–10). A distinct midrib, is, however, not to be found and the transverse section in this region (Fig. 10) indicates the arrangement of different layers of cells to be the same as mentioned above. There is no collenchyma below the epidermis. The vascular bundle of the midrib as also of those of other veins of the lamina is poorly developed, being represented by a few annual and spiral vessels in the xylem on the ventral side and a few phloem cells on the dorsal side. A distinct bundle sheath surrounds the vascular bundle of the midrib as well.

Hydrocotyle asiatica

Stem.—The transverse section of the stem (Figs. 12 and 13) shows on the outside a single-layered epidermis covered externally by cuticle. The epidermal cells are small, cubical, and convexly arched outward, measuring 14–18–22 microns × 10–14–17 microns. Following the epidermis is a collenchyma of 2–3 layers of cells which are more or less isodiametric, measuring 14–17–22 microns in diameter. The remaining part of the cortex is occupied by 6–7 layers of parenchymatous cells which are much larger than the collenchymatous cells and measure 25–38–50 microns in diameter. A distinct endodermis is, however, not easily made out. The cortex encloses a ring of seven vascular bundles. These have toward the periphery strongly developed, semilunar patches of sclerenchyma which are stained red by phloroglucin and hydrochloric acid. The vascular bundle is of the common, collateral, and open type. The phloem consists of sieve tubes with companion cells and phloem parenchyma and the xylem consists of wood vessels, tracheides, and xylem parenchyma. The protoxylem elements are arranged toward the inner side, the metaxylem toward the periphery. The vessels are 17–30–35 microns in diameter.

In the center is the pith, which has more or less rounded isodiametric cells with distinct intercellular spaces, measuring 34–50–70 microns in diameter. The pith joins the cortex by medullary rays which are characterized by the possession of resin canals, usually one in between two vascular bundles. Resin canals are found also in the cortical region. These are surrounded by a layer of 6–7 small, thin-walled epithelial cells and the central duct is 25–35 microns in diameter.

Petiole.—The petiole in transverse section (Figs. 14 and 15) has a characteristic outline, due to the sides projecting out from the ventral groove. The epidermis is covered externally by a cuticle and has its inner walls adjoining the cortex, greatly thickened. The cells are somewhat cubical and measure 15–17–20 microns × 10–15–18 microns. Beneath the epidermis is a collenchymatous ring of 2–3 layers of cells. The collenchyma is, however, absent on the sides of the projecting arms of the petiole



Leaf of *Hydrocotyle asiatica* Linn.

Fig. 14.—Transverse section of the petiole. × 40.

Fig. 15.—Details of the same. × 225.

Fig. 16.—Transverse section of the leaf. × 40.

Fig. 17.—Upper surface showing the distribution of stomata. × 225.

Fig. 18.—Lower surface showing the distribution of stomata. × 225.

Fig. 19.—Transverse section passing through midrib. × 225.

Fig. 20.—Details of the transverse section of the leaf. × 225.

Ep., epidermis; Collen., collenchyma; Paren., parenchyma; R.C., resin canal; U.Ep., upper epidermis; Pal., palisade layer; Spongy, spongy layer; L.Ep., lower epidermis.

(Fig. 14). These cells have great angular thickenings (Fig. 15) and are polygonal, measuring 17–25–34 microns × 17–22–30 microns. Following the collenchyma is a broad zone of parenchyma, the cells of which are more or less round, showing prominent intercellular spaces and measure 25–50–68 microns in diameter. Within this zone are to be found seven vascular bundles, two of which are present in the projecting arms of the petiole, while the remaining five form a dorsal arc. Usually there is a resin canal situated on the dorsal side of these vascular bundles. Some of the parenchymatous cells contain rosette crystals of calcium oxalate, measuring 8–15 microns in diameter.

Leaf.—The leaf has a structure of the typical dorsiventral leaf, with its mesophyll differentiated into an upper palisade and a lower spongy tissue (Figs. 16 and 20). The surface of the leaf is covered with a thin, striated cuticle which is more strongly developed on the midrib. Stomata occur on both the surfaces (Figs. 17 and 18). They are usually surrounded by three subsidiary cells, but occasionally four or five cells also accompany them. Of the three subsidiary cells, two are often placed opposite the stomatal aperture, more or less parallel, while the third is at right angles to the pore. The stomata are 28–31–34 microns long and 24–26–28 microns broad with the stomatal aperture being 6–10 microns wide. Hair is absent in this leaf. The distribution of stomata and the number of epidermal cells per sq. mm. leaf surface (Table II) indicate that the lower surface has greater numbers of these than the upper one.

TABLE II.—NUMBER OF STOMATA AND EPIDERMAL CELLS PER SQ. MM. LEAF SURFACE OF *Hydrocotyle asiatica*

Surface	Stomata	Epidermal Cells
Upper	104–140–218	744–805–845
Lower	180–220–243	825–956–1027

In surface view the epidermal cells are all polygonal with straight walls. They are bigger on the upper surface than on the lower one (Figs. 17 and 18) and measure 24–58–85 microns × 20–32–46 microns on the ventral side and 18–52–75 microns × 17–28–40 microns on the dorsal side.

In transverse section (Fig. 20) the epidermis has somewhat tangentially elongated cells which are larger on the upper side than on the lower one. They are 15–22–32 microns × 12–15–20 microns on the upper surface and 9–14–20 microns × 6–10–15 microns on the lower surface. Beneath the upper epidermis are 2–3 layers of palisade tissue. The upper palisade layer consists of larger cells, measuring 38–50 microns × 17–25 microns. The spongy mesophyll is represented by 5–7 layers of loosely arranged and somewhat isodiametric cells which are bigger in the upper layers with a diameter of 17–35 microns, while the lower layers possess a diameter of 12–18–28 microns. A few cells of the spongy layer also contain rosette crystals of calcium oxalate, measuring 8–15 microns in diameter.

The midrib has a characteristic structure in the transverse section (Fig. 19). The epidermal cells, particularly on the dorsal side, are cubical. Below the epidermis is found a collencyma of 2–3 layers of cells on both the sides, following which are 4–5 layers of parenchymatous cells, mostly devoid of chloroplasts. The central region is occupied by a well-developed vascular bundle with phloem toward the dorsal side and the xylem on the ventral side. As usual the phloem consists of sieve tube tissue with companion cells distributed in the phloem parenchyma, while the xylem is composed of radial rows of xylem vessels with intervening xylem parenchyma. No distinct bundle sheath surrounds the vascular bundle either of the midrib or of the lamina.

To elucidate further the diagnostic differences of these two species, their stomatal indices and palisade ratios have been studied (Tables III and IV).

TABLE III.—STOMATAL INDICES OF *Herpestis monniera* AND *Hydrocotyle asiatica*

	Herpestis monniera		*Hydrocotyle asiatica*	
	Upper Surface	Lower Surface	Upper Surface	Lower Surface
	12.77	17.25	20.51	17.32
	12.50	16.44	14.56	16.86
	13.76	17.35	11.58	17.26
	13.98	16.64	15.46	17.65
	15.96	15.78	14.45	16.57
	12.46	17.65	14.84	17.56
	14.11	17.20	14.83	17.56
	12.95	16.27	15.24	21.28
	11.50	16.02	14.32	17.64
	13.17	14.95	14.43	16.71
	12.76	14.75	16.12	15.92
	15.11	16.58	15.43	19.43
	14.34	17.45	14.56	18.25
	13.70	15.58	14.25	19.43
	13.38	17.65	13.46	19.43
	12.24	16.44	14.53	17.64
	13.23	17.25	15.27	21.05
	15.10	15.80	14.56	17.58
	12.05	14.95	15.25	19.56
	14.35	16.55	14.58	16.68
Mean	13.50	16.42	14.93	17.89

Analysis of Variance

Variation Due to	Degrees of Freedom	Sum of Squares	Mean Sum of Squares
Species	1	43.3777	43.3777
Surface	1	179.3065	179.3065
Surface × species	1	0.11025	0.11025
Error	76	152.34355	2.00452
Total	79	375.13800	

The analysis of variance shows that highly significant variation exists between these two species. Variation due to surface is also significant. This is because great differences exist in the distribution of stomata on the upper and the lower surfaces, per sq. mm. leaf area, particularly in the case of *Hydrocotyle asiatica*, where the lower surface happens to

possess at an average 50–60% more stomata than the upper one. The interaction between surface and species is not significant. These two species therefore can be distinguished easily by their stomatal indices, since *Hydrocotyle asiatica* possesses a much higher value than *Herpestis monniera* (Table V).

TABLE IV.—PALISADE RATIOS OF *Herpestis monniera* AND *Hydrocotyle asiatica*

Herpestis monniera		*Hydrocotyle asiatica*	
1.25	1.50	3.75	3.50
1.75	1.25	5.00	5.25
2.25	2.25	5.75	4.00
2.00	2.00	3.25	3.50
2.25	2.00	4.00	5.75
2.00	2.25	5.25	3.75
1.75	2.00	4.00	4.00
2.25	1.75	4.75	5.25
1.50	1.25	5.00	4.50
2.00	1.75	4.00	5.50
Mean 1.85		4.46	

Analysis of Variance

Variation Due to	Degrees of Freedom	Sum of Squares	Mean Sum of Squares
Species	1	68.2516	68.2516
Error	38	19.0834	0.5022
Total	39	87.335	

It will be seen from the above table of variance that very high significant variation exists between the two species. Thus they can be differentiated on the basis of their palisade ratios also.

TABLE V.—RANGE OF VARIATION IN THE STOMATAL INDICES AND PALISADE RATIOS OF *Herpestis monniera* AND *Hydrocotyle asiatica*

Species	Stomatal Indices: Upper Surface	Stomatal Indices: Lower Surface	Palisade Ratios
Herpestis monniera	11.5–13.6–15.9	14.9–16.3–17.7	1.25–1.75–2.25
Hydrocotyle asiatica	11.6–15.2–20.5	15.9–18.9–21.1	3.25–4.00–5.75

DISCUSSION

Herpestis monniera and *Hydrocotyle asiatica* belong to two different families. Accordingly they differ to a great extent in their anatomical features. In their habitat too these vary a great deal; *Herpestis monniera* grows in more moist places than *Hydrocotyle asiatica*, consequently it possesses such structures as are characteristic of plants growing in moist localities. The presence of air chambers and the absence of collenchyma or sclerenchyma in the stem, inadequate differentiation of mesophyll into palisade and spongy tissues in the leaf, and the reduction of vascular elements both in the stem and the leaf are some of the important characteristics which point out to its habitat which is more of a hydrophytic nature than of the mesophytic one. *Hydrocotyle asiatica*, on the other hand, shows somewhat xerophytic characteristics. The salient differences in their anatomical features which may help one to distinguish these two species are therefore the following:

Herpestis monniera	*Hydrocotyle asiatica*
Stem Structure	
1. Collenchyma is absent.	A well-developed collenchyma is present below the epidermis.
2. The cortex possesses large air chambers and is thus lacunar.	The cortex has only small intercellular spaces.
3. The stele is reduced and consists of a continuous ring of a few phloem and xylem elements.	The stele is broken up into 7 vascular bundles arranged in a ring.
4. Sclerenchyma is absent.	Sclerenchyma surrounds the peripheral side of each vascular bundle.
Leaf Structure	
5. Epidermal cells are wavy walled in surface view, with lower surface showing greater waviness.	Epidermal cells are straight walled and polygonal in surface view.
6. Stomata are present on both the surfaces. There is no definite arrangement of subsidiary cells; usually 3 or more cells surround the stomata. The epidermal cells and stomata are smaller and less in number per sq. mm. leaf area.	Stomata are found on both the surfaces, each surrounded by 3 or more epidermal cells. The number of stomata and of epidermal cells are greater per sq. mm. leaf surface.
7. Typical glandular hairs on both surfaces.	Hairs are absent.
8. Cuticle is strongly developed, occurring as prominent striations.	Cuticle is not so well developed, occurring as faint striations.
9. The mesophyll is poorly differentiated into palisade and spongy layers.	The mesophyll is well differentiated and there are 2–3 layers of palisade cells.
10. Occasionally a solitary crystal of calcium oxalate may be found in the spongy mesophyll.	Rosettes of calcium oxalate are found in the mesophyll.
11. Vascular tissue both in the lamina and the midrib is greatly reduced.	Vascular tissue is very much developed.
12. Collenchyma is absent in the midrib region.	Collenchyma is present below each epidermis.
13. Values representing stomatal indices and palisade ratios are lower. The range of stomatal indices lies between 11.50 and 17.65; palisade ratios between 1.25 and 2.25.	The stomatal indices and palisade ratios are much higher, the former varying between 11.58 and 21.05 and the latter between 3.25 and 5.75.

SUMMARY

The present investigation deals with the anatomy of the leaf and stem of *Herpestis monniera* and *Hydrocotyle asiatica*, both of which are known as *Brahmi*.

The stem of *Herpestis monniera* shows on the outside a layer of epidermis followed by a wide lacunar cortex. The endodermis surrounds internally a narrow zone of pericycle and a wide continuous ring of vascular tissue. In the center is found a small pith.

Characteristic glandular hairs and stomata are present on both the surfaces of the leaf of *H. monniera*. The epidermal cells are wavy walled. The differentiation of mesophyll into palisade and spongy tissues is poor and the vascular tissue is also much reduced. A distinct bundle sheath surrounds each vascular bundle.

The stem of *Hydrocotyle asiatica* consists of a single layer of epidermis, followed by a narrow ring of collenchyma and a broad region of parenchyma in which are distributed seven vascular bundles in a ring. A distinct endodermis is not to be seen. Each vascular bundle is surrounded toward the periphery by a semilunar patch of sclerenchyma. The vascular bundle is of the open, collateral type. Resin canals are found distributed in the cortex.

The leaf of *H. asiatica* is characterized by a petiole having more or less similar structure as noted in the case of the stem. Stomata are present on both the surfaces, but hairs are absent. The mesophyll is differentiated into 2–3 layers of palisade cells on the ventral side and 5–7 layers of spongy cells on the dorsal side. The midrib has a collenchyma below each epidermis.

These two species differ greatly in their stomatal indices and palisade ratios, *Hydrocotyle asiatica* possessing greater values than *Herpestis monniera*. Other important anatomical differences between the stems and the leaves of these species have also been indicated.

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Book Review

Inorganic Chemistry, by W. NORTON JONES. The Blakiston Company, Philadelphia, 1947, xii + 817 pp. + 49 pp. Problems and Questions. 17 x 23.5 cm. Price $4.25.

Just as newer developments in the field of chemistry are adding to and changing previous ideas, the author has endeavvored to revise the older order of presentation of a first year course in college chemistry. This has been done for the purpose of increasing student interest and to enable him to better grasp and retain the facts in the light of modern concepts of electronic structure and the periodic relationship of the elments.

For conservation of time on the part of the student, the treatment of the historical aspects is limited to brief sketches and portraits of those men and to those events which have been so important in shaping the course of the science of chemistry from a cultural and historical standpoint.

The theme followed by the author to maintain interest is a presentation of the theoretical aspects, interspersed with descriptive chapters and practical application.

Following each chapter bibliographies on the particular subject covered are included for the convenience of the student.

This volume should be interesting and valuable to the first year student in college chemistry either as a textbook or as an adjunct to the one in use.—NEULON DEAHL.

Thixotropic Mineral Gels and Their Therapeutic Possibilities*

By HERMAN J. SCHNEIDERWIRTH and PAUL W. WILCOX†

Some aspects of thixotropic behavior are discussed and experimentally developed thixotropic systems in water dispersions of alkaline earth metal salts are described. Factors influencing and producing thixotropy in these systems are discussed. The possible application of thixotropic gels as therapeutic agents is presented.

THE DISPERSION of small solid particles in a fluid sometimes results in the formation of a rigid gel when the mixture is at rest, but upon shaking the gel becomes a readily flowing suspension, called a sol. This transformation takes place under isothermic conditions and often is reversible at will for an unlimited period of time. Thixotropy is the name given to this physical behavior. It is called a "phenomenon" indicating that it is something beyond ordinary conception, something yet unexplained.

Some liquid dispersions of small mineral particles exhibit thixotropy naturally, but it is possible also to produce thixotropy artificially under experimental conditions. However, no definite rules exist to aid a research worker in establishing a given thixotropic system. This is strikingly demonstrated by Pryce-Jones (1) who states: "It may be safely assumed that at the present time no theory can explain the behavior of thixotropic systems or can predict the type of flow resulting from a dispersion of a given volume of fine particles in a fluid."

Several theories have been advanced to explain thixotropic behavior. One of them suggests that attracting and repelling forces of an electric nature acting upon or between the surfaces of the particles rule the phenomenon. These forces, some of which are believed to be London-van der Waals forces, compel the colloidal particles to arrange themselves toward each other in a certain pattern resulting in gel formation. As shown by Schalek and Szegvari (2) and by Hauser (3) in ultramicroscopic studies, the particles first lose their translatory, then their rotatory Brownian movements. Thereafter they are found to be evenly built into a semisolid gel structure and placed motionless face to face or end to end, separated by the dispersing medium. This peaceful arrangement easily is destroyed by agitation. Thereby, the particles are torn loose and slide along or tumble over each other. Everything is temporarily out of line. Brownian movement starts again and a readily flowing sol results. The latter is sustained for seconds or days, depending upon the thixotropic condition present. During this time the gel state gradually is being restored. Unfortunately, convincing experimental evidence of the theory outlined and of others advanced is lacking.

Thixotropy by no means is limited to solid particles of minerals in a fluid. Organic colloids, foams, and emulsions may be made to exhibit thixotropy (4). In short, the latter is a universal colloidal phenomenon, unlimited in scope. Complex, and as yet unexplained functions of living cells are influenced by thixotropic behavior (5). Among others, the appearance of neoplasm in parts of the body remote from the seat of a primary tumor may be due to the ability of cancer cells to exhibit thixotropy during some stages of their migration. A closer study of the thixotropic phenomenon in biocolloids may yield important clues to the understanding of many vital biologic processes. Finally, one can speculate that thixotropic behavior of colloids in an infinitesimally small dynamic way may possibly correlate with the electric theory of matter as applied to molecules and atoms. As the particle size of matter decreases, electric energy acting upon or between the particles

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increases until it reaches the tremendous proportions often encountered in subatomic phenomena.

This presentation is limited to experimentally produced thixotropic systems of aqueous dispersions of alkaline earth metal salts and their possible practical applications in pharmacy.

EXPERIMENTAL

Particle Size and Shape.—Past studies of strongly thixotropic systems made by different investigators (6) and (7) show that most favorable conditions are present when the particles have the following characteristics. They should be from submicronic to 5 microns in size, anisometric rather than isometric, and of plate or rod-like shape. We were not able to produce such particles by extensive mechanical treatment of aqueous dispersions of finely powdered mineral salts in a colloid mill. However, when aqueous solutions of alkaline earth metal salts such as calcium, magnesium, barium, and strontium chlorides were allowed to react under specific conditions with alkaline salts of phosphates, carbonates, and silicates, colloidal dispersions of the respective water-insoluble salts that by subsequent treatment could be converted into thixotropic gels were obtained. Particle size and shape were influenced mainly by the temperature at which the reactions took place. Agitation and rate of flow at which the solutions were combined were second in importance. The solutions were prepared in concentrations close to the saturation point at the respective temperatures. Approximately stoichiometric equivalent quantities of the reacting chemicals were used.

Fig. 1.—Dispersions obtained at 5°, 10°, 25°, 55°, and 90° after standing twenty-four hours at room temperature.

The following is an example, showing the preparation of a mixture of calcium and magnesium phosphates and carbonates at different temperatures and the degrees of dispersion of the particles obtained thereby.

Two solutions were prepared in the following manner:

Solution A

Calcium chloride, crystalline.....	122.25	Gm.
Magnesium chloride, crystalline..	6.40	Gm.
Distilled water, a sufficient quantity, to make.....	1000	cc.

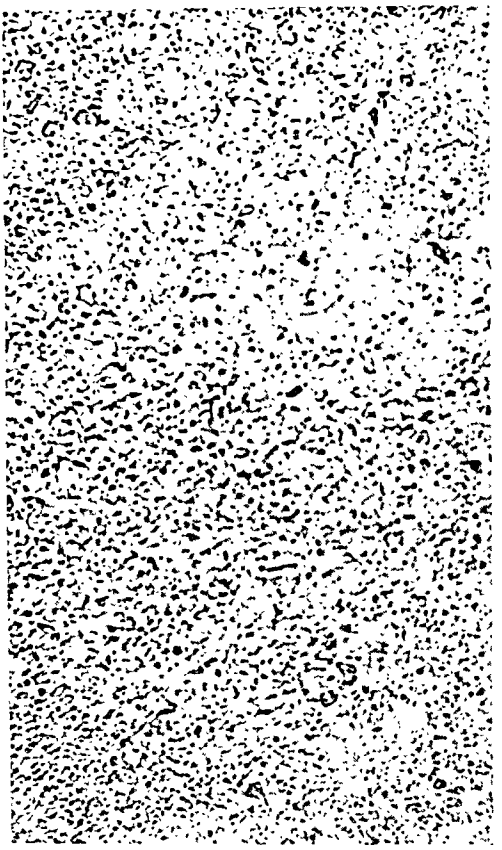
Solution B

Sodium phosphate, dibasic, anhydrous.....	108.00	Gm.
Sodium carbonate, monohydrated.	16.25	Gm.
Distilled water, a sufficient quantity, to make.....	2000	cc.

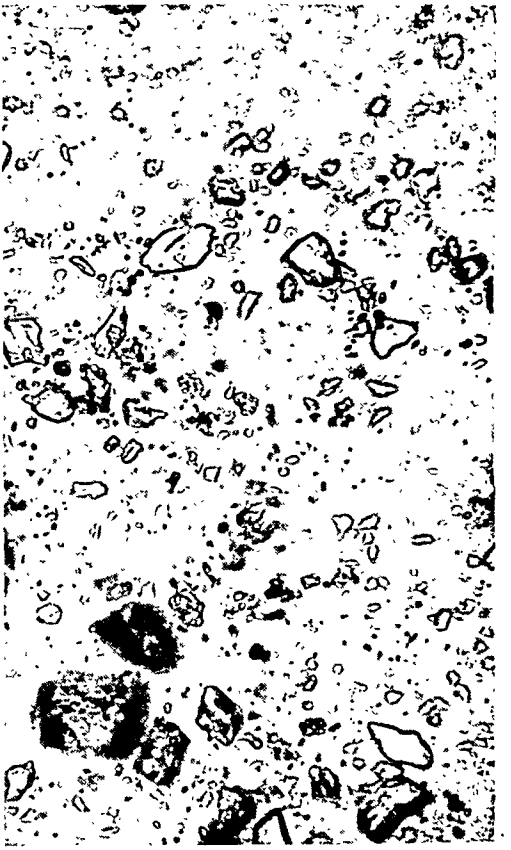
Both solutions were cooled to 5°. One hundred cubic centimeters of solution A was added to 200 cc. of solution B in a slow, even stream while agitating solution B. The voluminous precipitate was shaken for ten minutes and transferred in part to a graduated 100-cc. cylinder. The same procedure was repeated with the remaining solutions A and B after warming both to 10°, 25°, 55°, and 90°. The suspensions obtained under these conditions are shown in Fig. 1.

Conversion into Thixotropic Gels.—The supernatant liquids from the dispersions were decanted to remove the bulk of the water-soluble electrolytes formed during the chemical reaction. Thereafter filtration by suction, using a Büchner funnel, produced soft gels from which additional water was removed in a press. It was comparatively easy to obtain in this manner gels containing from 30% to 35% total solids. Of course, concentration may be regulated at will to meet specific requirements. The mineral gels prepared in this manner formed heavy, semisolid "cakes" that could be broken up and converted into thick pastes by mechanical mixing. Thereafter, sufficient citric acid to give a concentration of 0.5% to 1.5% was added and dissolved by mixing. This resulted in a slight softening of the pasty mass. The *p*H of such mixtures approximated 4.5.

When this *p*H was raised by the addition of an alkali such as sodium hydroxide, sodium carbonate, ammonia water, or an amine to a point above *p*H 5.0, the mixture was converted into a liquid sol that exhibited thixotropy. An increase of *p*H above 6.5 gradually caused the reconversion of the sol into a thick paste with loss of thixotropy. Thus, within a narrow *p*H range (approximately 5 to 6.5) a thixotropic system was established. The time of conversion from the sol to the gel state as well as the degree of viscosity of the sol and the rigidity of the gel varied with different alkaline earth metal salts. In most experiments reconversion took place within a few minutes and the sols were of comparatively low viscosity. They could be poured easily and would pass through a narrow gauge hypodermic needle without difficulty, whereas the subsequently formed gels were rather rigid in structure. It was further found that the *p*H range of thixotropic be-



Thixotropic Gel—415×



Emulsion—415×

Figure 2

havior could be broadened considerably, and thixotropy in general enhanced, when carbohydrates such as lactose and dextrose, or hexahydric alcohols such as sorbitol or mannitol, were added to the alkaline earth mineral gels and dissolved therein. Thus thixotropy was obtained within a *p*H range of from 4 to above 10.

Some Properties of the Thixotropic Mineral Gels.—The size and shape of particles in a thixotropic gel prepared from a dispersion obtained at a temperature of 55°, as described above, are shown in one of the photomicrographs of Fig. 2. As a comparison a photomicrograph of an emulsion that was prepared from an aqueous dispersion of finely powdered mineral salts having substantially the same composition as those present in the thixotropic gel is presented. The emulsion was prepared in the following manner:

Calcium phosphate, dibasic, fine powder	174 Gm.
Calcium carbonate, precipitated, fine powder	16 Gm.
Magnesium phosphate, dibasic, fine powder	5 Gm.
Magnesium carbonate, light, fine powder	5 Gm.
Cod-Liver Oil, U. S. P.	200 cc.
Acacia, fine powder	50 Gm.
Distilled water, *q. s.*	1000 cc.

The acacia powder was added to the cod-liver oil and dispersed by trituration. One hundred cubic centimeters of distilled water was added and the mixture was emulsified. The combined calcium and magnesium salts were mixed with 450 cc. of distilled water and treated in a colloid mill for twenty minutes. This dispersion was then mixed with the emulsion, adjusted to 1000 cc., and twice put through a hand homogenizer. The procedure is similar to the one given in "The Pharmaceutical Recipe Book," ed. 3, page 44, for compound emulsion of magnesium trisilicate and kaolin. However, the product prepared by us was superior on account of the additional colloid mill and homogenizer treatment. Cod-liver oil was used instead of mineral oil in order to prevent a possible interference with calcium absorption in animal tests which are described herein and in which tests this emulsion was used.

The absorption and utilization of calcium from the thixotropic gel in comparison with the emulsion described were determined in preliminary *in vivo* tests on 50 rats in the following manner:

Fifty young albino rats of 85 Gm. average weight were kept on a calcium-free diet for three weeks. Thereafter, one group of 15 rats was fed by stomach tube 1 cc. of a thixotropic gel prepared as described above. Another group of 15 rats was fed 1 cc. of the emulsion described above. After one, two, and five hours, 5 rats of each group were sacrificed and the whole intestinal tracts were dissected and reduced to ash. Four unfed control rats were treated in the same manner.

Calcium Absorption

1. Thixotropic Gel

	Found in Intestine	Absorbed
Intake (per rat)..	64.70 mg.	.....
1 hr.....	63.53 mg.	1.17 mg.
2 hr..... ..	62.96 mg.	1.74 mg.
5 hr.....	55.24 mg.	9.46 mg.
2. Emulsion		
Intake (per rat)..	57.50 mg.	.....
1 hr.....	59.30 mg.	None
2 hr.....	58.94 mg.	None
5 hr.....	51.28 mg.	6.22 mg.
3. Unfed Controls.	1.05 mg.	.....

Of the remaining 16 rats two groups of 6 rats each were fed daily 1 cc. of the two different products for four successive days. Four rats served as controls. On the fifth day these rats were sacrificed and the intestinal tracts were dissected out and discarded. The animals minus intestinal tracts were reduced to ash and the calcium in the ash residue was determined.

Calcium Utilization

1. Thixotropic gel fed to 6 rats:
   Intake (per rat).....64.70 mg. Ca daily for 4 successive days
   Average found in 6 rats minus intestines.....366.95 mg. Ca—gain 119.10 mg. (corrected for weight)
2. Emulsion fed to 6 rats:
   Average intake.....57.50 mg. Ca daily for 4 successive days
   Average found in 6 rats minus intestines.....325.11 mg. Ca—gain 85.01 mg.
3. Unfed Controls:
   Average found in 4 rats minus intestines.....240.10 mg. Ca.

The figures obtained are statistically significant enough to conclude that the thixotropic gel was definitely better absorbed from the intestinal tract of rats and better utilized by their body than was the emulsion. Since the number of animals in each group was rather small, no attempt is being made to evaluate the advantages of one product over the other in a percentage form. Such figures we hope to be able to present after completion of further animal tests conducted on a larger scale.

DISCUSSION

Since this research was aimed at a possible application of the thixotropic gels as therapeutic agents, a critical evaluation of the practical advantages gained seems to be in order.

Substantially aqueous, fluid medications containing fine, solid particles immiscible with or insoluble in water usually are prepared in the form of suspensions or emulsions. Often there is a separation of the liquid phase upon standing. Sedimentation and coagulation of the particles into a lumpy precipitate impossible to redisperse by shaking is another common occurrence. Further, sometimes the use of large amounts of viscous, gummy or oily substances, needed to obtain permanent suspension, limits the concentration of therapeutically active ingredients. These admixtures also often coat the particles to a degree that may interfere with their absorption and utilization by the body. These products contain an abundance of uneven, sharp-edged, gritty particles whose unpleasant taste can be masked only feebly by the skill of the pharmacist. In such systems the particles are in an uncontrollable, unorganized state. Some of them show Brownian movement, and others simply follow the law of gravity and sink to the bottom.

Thixotropic gels in contrast are controllable, regimented systems. The disadvantages inherent in the comparable forms of application as outlined above largely are overcome. Concentrations of from 30% to 40% of fine solid particles may be incorporated into the semisolid gels, yet the sols pour easily. Such concentrations cannot be obtained in pourable mechanical suspensions or emulsions.

Stability of the thixotropic gels is another important gain. Separation of the liquid phase does not take place except for a slight syneresis common to all gels and caused by shrinkage after standing for several months. Smoothness and creamy consistency, due to fine particle size and to thixotropy itself, is another practical advantage for oral preparations. Finally, evidence for increased absorption and utilization of calcium from a thixotropic gel as compared with an emulsion when both were fed to calcium-starved rats have been shown in the experimental portion of this communication. It is at least possible that similar results can be obtained in human subjects.

To obtain optimum therapeutic response in general mineral deficiencies, additional mineral elements known to be needed by the body, as described in a recent review by Richards (8), were incorporated in some of our experimental lots. Such additions did not interfere with stability or other characteristics of the thixotropic gels.

Another logical step was the addition of vitamins. The writers have prepared many such products and find that fat-soluble vitamins such as vitamin A, vitamin D, and α-tocopherol, as well as the water-soluble vitamins such as ascorbic acid and members of the vitamin B complex, remain evenly dispersed

throughout the thixotropic gels. Losses in vitamin potency after standing for twelve months were moderate and within expected limits. This is understandable when one realizes that the vitamins are in a state of complete rest, imprisoned in a solid gel in the presence of antioxidants which had been added to enhance stability.

SUMMARY

1. Some aspects of the present status of our knowledge of thixotropic behavior are discussed briefly.

2. Experimentally developed thixotropic systems in aqueous dispersions of alkaline earth metal salts are described. The factors influencing and producing thixotropy in these dispersions are particle size and shape, concentration, *p*H and the presence of citrates, carbohydrates, or hexitols.

3. Possible applications of the prepared thixotropic gels in mineral therapy and their use as adsorbents or vehicles for therapeutic agents, are presented. The advantages of such medications are: high concentration of ingredients in a gel-sol form that is reversible at will; stability; freedom from grittiness; controlled, even distribution of ingredients throughout the gels; and possible increase in therapeutic response.

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Iodine Research Award Nominations Requested

Nominations are now being received by the AMERICAN PHARMACEUTICAL ASSOCIATION for the Iodine Educational Bureau Award recognizing outstanding research in the chemistry and pharmacy of iodine and its compounds as applied in pharmacy or medicine. Any member of the ASSOCIATION may propose a nominee by submitting specific identification of the work to be considered in the competition, a biographical sketch of the nominee including date of birth, and a list of his publications. Eight copies of the nomination must be submitted to the Secretary of the AMERICAN PHARMACEUTICAL ASSOCIATION, Dr. Robert P. Fischelis, 2215 Constitution Ave., N. W., Washington 7, D. C. To be eligible for the 1948 Award, nominations must be received before January 1.

Establishment of the new iodine award was announced at the Milwaukee convention of the AMERICAN PHARMACEUTICAL ASSOCIATION, which will administer the competition.

A nominee must be a resident of the United States or Canada. He must have accomplished outstanding research in the chemistry or pharmacy of iodine and its compounds as applied in pharmacy or medicine.

During the period covered by the nomination the nominee shall have been actively engaged in, shall have completed, or shall have published a report upon the line of investigation for which the award is made. During a period of two years prior to the date of nomination, the nominee shall not have been engaged in research under the sponsorship of the Iodine Educational Bureau, Inc.

The award consists of $1000 and a diploma setting forth the reasons for selection of the recipient. It may be presented biennially at the annual meeting of the AMERICAN PHARMACEUTICAL ASSOCIATION.

The recipient will deliver a paper or lecture upon the subject of his scientific work at the meeting at which the award is conferred. His paper, or address, will then be published in the JOURNAL of the AMERICAN PHARMACEUTICAL ASSOCIATION. In addition to the sum of the award, the recipient will receive an allowance of not more than $250 to defray his expenses in attending the meeting.

The recipient will be selected by an award committee which is appointed by the chairman of the ASSOCIATION's Council and which functions under prescribed rules.

Should the award committee fail to find a suitable recipient in any biennium, two awards may be made during the next biennium in the same or successive years, if suitable recipients are selected.

Lubricants in Compressed Tablet Manufacture*,†,‡,§

By J. EDWARD WOLFF,‖ H. GEORGE DEKAY, and GLENN L. JENKINS

A theoretical discussion of the possible causes for sticking and binding during compression of granulations is presented together with fundamental research on the mode of action of a lubricant. One hundred seventy-one water-soluble compounds were studied for lubricating properties. Two per cent of a compound lubricant composed of equal parts of Dreft, sodium oleate, and Maprofix gave satisfactory lubrication to 72% of the tested granulations.

THE MANUFACTURE of tablets, as we know them today, has recently had its one hundredth anniversary. There has been little change in the list of lubricants appearing throughout this period and today we still find talc, the stearates, mineral oil, and boric acid commonly employed.

A lubricant is generally defined as a substance which is added to the granulation to insure uniform feeding into the die and to prevent the material from adhering to the punches and die during and after compression.

The exact manner in which a lubricant accomplishes these important functions is not clearly understood. It is known that a compound may offer lubrication to one granulation, while it fails to lubricate another. For some substances no lubricant is required, although generally it is necessary to add one before compression. The amount of lubricant used will vary, and be dependent upon both the granulation and the lubricant selected.

The questions arising are: Why do some substances require a lubricant, and why does a lubricant appear to show specificity in action?

This paper attempts to disclose some theoretical material published in allied fields of work which may in time bring forth answers to our questions. The thesis is divided into two portions, Part I being devoted to exploring the mode of action of a lubricant and Part II consisting of a study of water-soluble compounds for lubricating properties.

THEORETICAL DISCUSSION

Rodwell (1) reports that the lack of sufficient lubricant will result in enough friction to cause the tablet to crack. Husa (2) states that more lubricant is needed where there are excessive fines since fine powder has a greater tendency to stick to the face of the punch.

Various physical and chemical factors may enter into the causes for picking, sticking, and binding during compression. Peddie (3) points out that many substances have their melting points lowered with an increase in pressure. The migration of alkaloids to the surface of a tablet during compression suggests some electrical phenomena. Richards, *et al.* (4) state that volatile substances are more compressible than nonvolatile, as the nonvolatile compounds are already under internal pressure. Richards and Mathews (5) conclude that compressibility is large when the surface tension is small, and that the density is greater in compounds with a large surface tension and a small compressibility.

The conversion of sodium bicarbonate to sodium carbonate under pressure has been reported (6). Spring (7) states that bodies remain solid under compression unless they occupy a smaller volume in the liquid than in the solid state. Hallock (8) asserts that the melting point and liquidity of a product are as important as its constituents in determining the possibility of a chemical reaction under pressure. Johnston and Adams (9) observed three layers in a compressed body: the vitreous outer surface, suggesting the flow of a viscous liquid under pressure, a granular layer directly beneath the vitreous surface, and a matted crystalline layer within.

Creelman and Crocket (10) refer to the work of Tait at the University of Edinburgh where his special galvanometer recorded that the fall in temperature on relieving pressure is considerably less than the rise on the application of pressure.

Richards (11) made studies on electrification by impact whereby frictional electricity was compensated. The charge produced was directly proportional to the velocity of the piston and there was no immediate limit to the charge for most solid dielectrics.

* Received June 26, 1947, from Purdue University, School of Pharmacy, Lafayette, Ind.
† Presented at the meeting of the American Association for the Advancement of Science, Pharmacy Subsection, Boston, Mass., Dec. 28, 1946.
‡ Abstracted from a thesis presented by J. Edward Wolff to the Graduate Faculty of Purdue University in partial fulfillment of the requirements for the degree of Doctor of Philosophy, Sept., 1945.
§ This work was made possible through the research grant of Sharp and Dohme, Inc., Philadelphia, Pa.
‖ Present address: Medical Research Division of Sharp and Dohme, Inc., Glenolden, Pa.

EXPERIMENTAL

Part I

Experiment 1.—To determine the most effective way of incorporating the lubricant, fourteen water-soluble compounds were selected. Each was divided into three parts. The first portion was screened through a 120-mesh sieve and dusted on the granulation. The second portion was dissolved in water to make a nearly saturated solution and this solution was incorporated with the granulation by tumbling. The third portion was likewise dissolved in water and the solution was sprayed on the granulation. In the second and third portions the granulation was air dried for three days prior to compression. Aspirin-Starch Granulation (Dow) was the test granulation. The lubricants applied in solution form were found to be the most effective.

Experiment 2.—Sixteen granulations were studied for general structural arrangement under a hand lens magnifying 14 diameters and a compound lens magnifying 180 diameters. Compression studies with the Colton single punch machine revealed that there was no correlation between structure and compressibility of the screened granulations, as granules composed of masses of rectangular crystals were just as compressible as those composed of cubical crystals, or those having no common structural arrangement. In all tests, those granules having numerous interstices were most compressible.

Relative potential studies, using a properly grounded electroscope, showed that the colored boric acid carrying a positive charge separated in only 32% of the tests when the granulation carried a negative charge, whereas 75% of the tests showed separation when the granulation carried a positive charge. Compression studies using the Colton single punch and the Stokes RB-2 rotary machines revealed that the degree of adherence of the lubricant to the granules does not appear to have any influence on the ability to lubricate the granules during compression, and screening out the free colored lubricant did not alter compression.

Experiment 3.—The fact that crystalline substances, having no center of symmetry, show piezoelectric properties and are capable of producing electric charges when they are compressed, led to the opinion that possibly, during the rapid compression of some compounds, a charge, too large to be carried off by the grounded machine, is built up. A lubricant might act as a conductor by providing more points of contact or it might act as an insulator. Resistance to the passage of a current creates heat, heat causes substances to melt, and the melting points of some substances are lowered under pressure. The adhesive nature of most granulations would be likely to cause binding or sticking, should sufficient heat be generated.

A device was made (Fig. 1) whereby a compound could be placed in an insulated cylinder, pressure applied, and the resistance to the passage of a current measured on a Megohm Bridge (Type 544-B No. 99 General Radio Corporation).

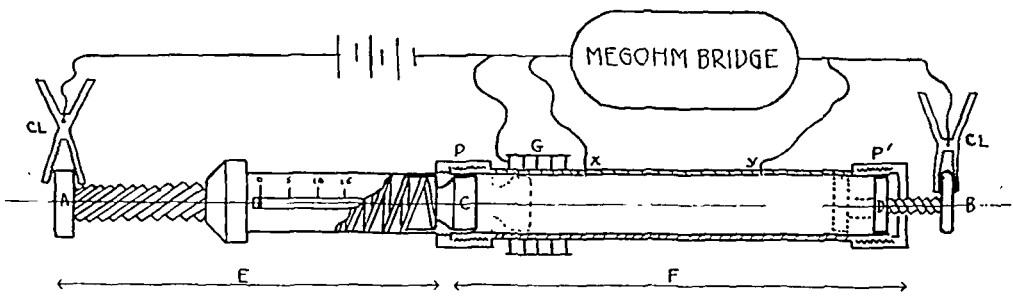

Figure 1

Key:
E—Monsanto hardness tester
F—Bakelite cylinder
G—Soft lead guard and ground
P and *P'*—Metal caps
CL—Clamp

It was impossible to observe the appearance of a lubricant on a granulation when both were white. Boric acid was selected as the lubricant. It was colored with a 10% solution of Amaranth, dried, pulverized, and sifted through a 120-mesh sieve. The colored lubricant was added to the granulation and thoroughly mixed by tumbling.

Microscopic study revealed that the lubricant becomes lodged in the roughened cavities of the granules and does not appear to envelop the granules (12). Although nearly all of the granules possessed rough edges, in 56% of the samples the lubricant did not adhere and a considerable amount of colored lubricant remained on paper after thorough mixing.

All compounds were introduced so that packing was as near uniform as possible throughout the tube *F*. Pressure was applied to the brass piston *C* until 15 Kg. was recorded. Points *x* and *y* were exactly 8.2 cm. apart and the diameter was 2.54 cm. A current of 120 volts was introduced and the resistance between points *x* and *y* read directly on the Megohm Bridge. The apparatus was grounded through the Megohm Bridge.

Wet granulations were made from which samples were removed at various stages of drying. Each

sample was studied for compressibility and moisture content and specific resistivity. The resistivity increased considerably as the moisture content decreased. None of the nonlubricated granulations was compressible with a moisture content greater than 4%. Each of the dry granulations offered a resistance of over 50,000 megohms to the passage of a current of 120 volts.

The resistivity to conductivity was determined on thirty-six dried and powdered compounds suggested as lubricants. In all but eight the resistivity was high. Those offering the least resistance to the passage of the current were: bentonite, magnesium trisilicate, Margel, Arctic Syntex M, magnesium silicate, sodium borate, Kelgin, and Tegin.

The action of a conductor such as bentonite on a granulation which offered a resistance of over 50,000 megohms was studied under a pressure of 15 Kg. From 1% to 5% bentonite was employed in several tests and in all tests the resistance did not change. When a static charge of 300 volts was placed on the compressed material, however, the charge fell off rapidly whereas a static charge placed on the compressed nonlubricated granulation remained with only a gradual loss of charge.

Experiment 4.—Beery (13) states that the lubricant plays an important part in the disintegration of a tablet.

To show this effect, the standard Aspirin-Starch Granulation (Dow) was prepared by screening through a number 16 mesh sieve, rejecting the fines passing through a number 30 sieve. Thirty-six compounds were selected as lubricants representing wetting agents, soaps, chemicals, and food products. Each was screened through a number 120 sieve, mixed thoroughly with the granulation, and compressed. All granulations were compressed with the Colton 2B single punch machine. Each tablet weighed 6 gr. Ten tablets had an average hardness of 3.5 Kg. when tested.

The food products representing flours and starches caused the tablets to disintegrate most rapidly followed by the wetting agents, chemicals, and finally the soaps. It must be noted, however, that the food products were employed in the largest amounts, followed by wetting agents, soaps, and finally chemicals.

Part II

Experiment 1.—The need for a water-soluble lubricant in the manufacture of compressed tablets has long been recognized, particularly if it is intended that the tablet give a clear solution when dissolved n water.

One hundred seventy-one water-soluble compounds were selected from organic and inorganic compounds and tested for lubricating properties. Solid substances were sifted through a number 120 sieve and incorporated with the granulation by tumbling. Liquids were added and mixed with the granulation by tumbling. One per cent of the lubricating compound was used, because it was found that 1% magnesium stearate offered satisfactory lubrication to the standard granulations, and the smallest possible effective amount of lubricant was desired. The standard granulations were: (*a*) aspirin compound, (*b*) calcium lactate, (*c*) vitamin C, and (*d*) dextrose.

Each granulation was made in sufficient quantity to carry through the entire experimentation and kept in closed containers. The density and moisture content of the granulations were determined at frequent intervals and were found to remain constant. Each granulation was screened prior to lubrication. The initial compression studies were made with the Colton 2B machine using a $^7/_{16}$-inch die and curved punches. Compression immediately followed lubrication.

The die and lower punch were removed, examined, washed, and dried before the next sample was compressed. Those compounds offering satisfactory lubrication were then incorporated with the respective granulation and compressed with the Stokes RB-2 rotary machine. In practically every test more lubricant was required when using the rotary than when using the single punch tablet machine although the granulations, the size of the die, and the tablet hardness were the same.

Combinations of all the satisfactory lubricating compounds were made and tried on the standard granulations, and samples of the lubricated granules were compressed using both the single and rotary punch machines. A compound composed of equal parts of Dreft (Procter and Gamble), sodium oleate, and Maprofix (Onyx Oil and Chemical Company) gave good results when 2% of lubricant was employed with twenty-nine different granulations which included twelve commercial samples.

SUMMARY AND CONCLUSIONS

A theoretical discussion of the possible causes for sticking and binding during the compression of granulations is presented together with fundamental research on the mode of action of a lubricant. One hundred seventy-one water-soluble compounds were studied for lubricating properties. Two per cent of a compound lubricant composed of equal parts of Dreft, sodium oleate, and Maprofix gave satisfactory lubrication to 72 per cent of the granulations studied.

The conclusions which may be drawn from this initial work include:

1. Most of the lubricants employed in tablet manufacture are insoluble in water and appear to be more effective than water-soluble lubricants.

2. A lubricant does not envelop a granule but becomes lodged in the roughened cavities of the surface.

3. A lubricant may play a part in the conductance of an electric charge produced during compression as a static charge passed off more readily from some of the lubricated granulations than from others and much more rapidly from a lubricated than from a nonlubricated granulation.

4. A lubricant applied in solution form is more effective than a powder dusted on the granulations.

5. The type of lubricant plays a part in the disintegration of a tablet since food products generally caused tablets to disintegrate more rapidly than wetting agents, soaps, or chemicals.

6. The compound lubricant composed of equal parts of Dreft, sodium oleate, and Maprofix offered satisfactory lubrication for 72 per cent of the tested granulations.

REFERENCES

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(2) Husa, W. J., "Pharmaceutical Dispensing," ed. 2, Husa Bros., Iowa City, Iowa, 1941, p. 93.
(3) Peddie, W., *Proc. Roy. Soc. Edinburgh*, **13**, 155(1885).
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United States Pharmacopœial Convention

Seventh Annual Financial Report, Covering the Period from May 1, 1946, to April 30, 1947, and Based Upon the Report of the Auditor

STATEMENT OF INCOME AND EXPENSE FOR THE YEAR ENDED APRIL 30, 1947

Income			
Sale of Pharmacopœias:			
Collections		$40,558.50	
Less: Decrease in Accounts Receivable			
April 30, 1946	$ 5,411.00		
April 30, 1947	890.75	4,520.25	$36,038.25
Sale of Reference Standards, A. M. A. Articles, etc.:			
Collections		$ 6,402.45	
Less: Decrease in Accounts Receivable			
April 30, 1946	$ 916.50		
April 30, 1947	803.00	113.50	
		$ 6,288.95	
Less: Decrease in Collections Pending Remittance to the Treasurer			
April 30, 1946	$ 719.25		
April 30, 1947	487.75	231.50	6,057.45
Interest on Investments:			
Collections		$ 2,630.74	
Less: Amortization of Bond Premiums		80.05	2,550.69
Miscellaneous Income:			
Collections			411.79
Total Income			$45,058.18

Less:				
Expenses				
Publication and Sales Expense		$14,930.30		
Less: Increase in Inventory				
April 30, 1947	$6,475.99			
April 30, 1946	4,014.59	2,461.40	$12,468.90	
Administration			4,592.72	
Revision		$33,646.16		
Less: Increase in Inventory Reference Standards				
April 30, 1947	$6,033.75			
April 30, 1946	2,073.02	3,960.73	29,685.43	
Research			11,474.58	
Stationery and Supplies—Inventory Adjustment		(Minus)	85.38	
Provision for Depreciation:				
Building		344.91		
Furnishings and Equipment		302.06	646.97	58,783.22
Excess of Expense Over Income For the Year Ended April 30, 1947				$13,725.04

STATEMENT OF CASH RECEIPTS AND DISBURSEMENTS (GENERAL ACCOUNT) FOR THE YEAR ENDED APRIL 30, 1947

Cash on Deposit, April 30, 1946		$62,414.24
Add:		
Receipts		
Sale of Pharmacopœias	$40,558.50	
Sale of Reference Standards	6,402.45	
Interest on Investments	2,630.74	
Use of Text by Others	10.00	
Miscellaneous	401.79	50,003.48
		$112,417.72

Less Disbursements	Publications and Sales	Administration	Revision	Research	Total
Printing and Binding	$14,930.30	.....	.....	.....	$14,930.30
Salaries and Honoraria	.....	$1,700.00	$13,500.00	.....	15,200.00
Clerical	.....	1,148.80	7,998.50	$ 129.90	9,277.20
Meetings	.....	583.42	1,207.13	3,443.29	5,233.84
Supplies	.....	58.00	1,343.24	785.32	2,186.56
Postage and Telegrams	.....	85.20	1,043.86	31.36	1,160.42
Compensation	.....	.....	.....	7,070.59	7,070.59
Headquarters	.....	.....	1,289.95	.....	1,289.95
General	.....	1,017.30	7,263.48	14.12	8,294.90
	$14,930.30	$4,592.72	$33,646.16	$11,474.58	$64,643.76

Add:		
Purchase of Furnishings and Equipment	$ 9.90	
Building Improvements	117.50	$64,771.16
Cash on Deposit, April 30, 1947		$47,646.56

A MORE DETAILED AND SUPPLEMENTARY STATEMENT OF THE REVISION AND RESEARCH EXPENSES

REVISION—MEETINGS

Chairman's Conferences—Transportation, Hotel, Meals, Etc., for the Revision Chairman and Others, for Meetings and Conferences Related to or Required by the Revision Program and Called by the Chairman	$ 1,207.13

REVISION—SUPPLIES

Stationery	$ 275.75
Mimeograph	594.99
Miscellaneous	472.50
	$ 1,343.24

REVISION—SALARIES

Revision Chairman, E. Fullerton Cook	$ 9,000.00
Executive Assistant, Adley B. Nichols	4,500.00
	$13,500.00

REVISION—GENERAL

Reference Standards	$ 3,320.75
A. M. A. Convention Exhibit	625.96
Inter Society Color Council	25.00
American Pharmaceutical Association—1945 and 1946	2,000.00
Receiver of Taxes, Philadelphia	1,180.15
Miscellaneous	111.62
	$ 7,263.48

HEADQUARTERS

Janitor's Salary (One-half)	$ 795.00
Utilities	365.90
Maintenance—Supplies	53.16
Repairs	29.70
Furniture and Equipment	26.19
Insurance	20.00
	$ 1,289.95

RESEARCH

Subcommittees	Meetings, Supplies, Postage, Clerical Travel and General	Technical Assistance	
1. Scope		Assistant to Dr. Youngken for Study on Rhubarb	$ 70.59
Meetings	$2,239.46	Assistant to Dr. Krantz	2,500.00
Miscellaneous	10.20	Assistant to Dr. Beal	4,500.00
5. Botany & Pharmacognosy	28.50		
7. Inorganic Chemicals	300.44		
8. Organic Chemicals	500.00		
13. Ointments, Etc.	466.46		
Amino Acids Committee	489.07		
Anti-anemia Board	121.56		
Sterile Products Board	58.23		
Vitamin Board	190.07		$ 7,070.59
	$4,403.99		4,403.99
Total			$11,474.58

Notes

Notes on the Role of Hydrogen Ion Concentration and Buffer Systems in the Preparation of Ophthalmic Solutions*

By HARRY W. HIND, FRANK M. GOYAN, and T. W. SCHWARZ

THE PAPER entitled, "A New Concept of the Role of Hydrogen Ion Concentration and Buffer Systems in the Preparation of Ophthalmic Solutions," by Hind and Goyan (1) has created sufficient interest to show the need for further clarification of at least two points.

The first has to do with the choice of preservative, especially in connection with "Solution 3 for Preparation of Eserine Solutions" (1). When this solution is used, a salicylate-benzalkonium incompatibility[1] similar to the nitrate-zephiran incompatibility reported by Huyck and Davy (2) cannot be avoided except by choosing some salt of eserine other than the salicylate. A more generally useful solution for eserine can be made by substituting phenylmercuric nitrate (1:100,000) for benzalkonium chloride (1:50,000). A further improvement results from substituting sodium sulfite for sodium metabisulfite as the antioxidant. The slow oxidation of the bisulfite by oxygen of the air causes a decrease in pH which promotes the formation of turbidity upon the addition of eserine salts to old solutions. Although no solution containing sulfite or bisulfite should be kept indefinitely, the substitution of sodium sulfite eliminates some of the undesirable effects of aging. The following formula embodies the changes suggested above.

SOLUTION FOR THE PREPARATION OF ESERINE SOLUTION

Boric acid, C. P. (22.0 Gm./L.)	0.356 *M*
Sodium sulfite (Na_2SO_3)	0.1%
Phenylmercuric nitrate	1:100,000
Distilled water	*q. s.*

The second matter that requires clarification has to do with the ambiguous column heading, "Grams of Sodium Chloride per 100 Cc. to Render Isotonic." In Table V (1) values tabulated under this heading for the Sørensen phosphate buffer system represent the calculated osmotic equivalent of each buffer mixture, expressed as grams of sodium chloride per 100 cc. of water. These values were calculated from the equation,

$$W = 17(L_1m_1 + L_2m_2),$$

which was derived by assuming that contributions to the freezing-point lowering from different solutes in a solution isotonic with blood serum are approximately additive (i.e., $\Delta T_f = L_1m_1 + L_2m_2 = 3.44\ W/58.45$). In these equations, ΔT_f represents the freezing-point lowering of a solution containing two solutes having *molal* freezing-point lowerings of L_1 and L_2, and molal concentrations of m_1 and m_2, respectively. This solution is isotonic with a solution of sodium chloride ($m = W/58.45$; $L = 3.44$) containing W Gm. of salt per 1000 Gm. of water. Values of L_1 and L_2 for different substances, and the significance of these values, are given by Goyan, Enright, and Wells (3). For the Sørensen Buffer mixture containing 90 cc. of $^1/_{15}M$ NaH_2PO_4 and 10 cc. of $^1/_{15}M$ Na_2HPO_4, $m_1 = (0.9/15)$, $m_2 = (0.1/15)$, and L_1 and L_2 have the values 3.22 and 4.22, respectively. The osmotic equivalent tabulated for 100 cc. of this buffer mixture should be one-tenth of the value of W, or 0.38 Gm. of sodium chloride. This is in complete agreement with the value given by Wells (4) and by Hind and Goyan (1), but to clarify this matter in the latter paper, the last column in Table V dealing with the Sørensen phosphate buffer might have been headed: "Osmotic Equivalent of Each Buffer Mixture Expressed as Grams of NaCl per 100 Cc. of Solution. (To render 100 cc. of solution isotonic, add an amount of NaCl equal to the difference between the tabulated value and 0.90 Gm.)"

Conversely, values tabulated for the Palitzsch borate buffer in Table IV (1) under the heading, "Grams of Sodium Chloride per 100 Cc. to Render Isotonic," represent weight of sodium chloride to be added to 100 cc. of the corresponding buffer solutions. These values were calculated by subtracting, from 0.90, one-tenth of the values calculated for W by the use of the equation given above. In view of the complex equilibria involved, the molal freezing-point lowerings for boric acid and for borax were assigned the values 2 and 7, respectively.

In calculations of the type illustrated above, molal and molar concentrations may be used interchangeably, and the computed weights of sodium chloride may be rounded off to one significant figure; greater accuracy is of no therapeutic significance. Other values given by Hind and Goyan may be rounded off to advantage. For example, a 0.2 *M* solution of boric acid, intended either for use with the Atkins and Pantin or the Gifford buffer, is properly made by dissolving 12.4 Gm. of boric acid in distilled water to make 1 L. of solution.

As a matter of possible academic interest, but of

* Received July 1, 1947 from the Barnes-Hind Laboratories, Inc., 430 Post St., San Francisco, and the University of California, College of Pharmacy, San Francisco.

[1] Preliminary experiments show that the boric acid-sulfite solution for eserine, containing any one of several cationic bactericidal quaternary ammonium salts such as Zephiran, Phemerol, and Emulsept, at a dilution of 1:50,000, slowly develops opalescence at room temperature with as little as 0.1% of sodium salicylate. Higher concentrations of sodium or eserine salicylate produce marked turbidity in a few minutes. Bromides and iodides may also form turbid solutions with benzalkonium salts, depending upon concentration. The same solution (without salicylate) can be used to make clear solutions of eserine hydrochloride. Further study is in progress. The need for this study was first brought to our attention by P. G. Devine, U. S. Shea, and W. C. Clark of the Barnes-Hind Laboratories, Inc.

no therapeutic significance, it should be noted that the freezing-point depression calculated for "Buffer Solutions for Group 2 Drugs" (1) is 0.50°, corresponding to an approximately 0.85% solution of sodium chloride. The value of 0.56° corresponds to a 0.95% solution of sodium chloride, a concentration often assumed to be isotonic with blood serum. In the same table, the formula for disodium phosphate is in error by the transposition of a subscript. Another typographical error of minor importance has been noted: the modified Palitzsch buffer is correctly given in Table IV (1), but the buffer range mentioned in the text (12 lines below the table) should have been given as *p*H 7 to 9.

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(3) Goyan, F. M., Enright, J. M., and Wells, J. M., THIS JOURNAL, 33, 74(1944).
(4) Wells, J. M., THIS JOURNAL, *Pract. Pharm. Ed.*, 5, 99 (1944).

A Note on the Synergism of the Central Nervous System Stimulants*

By RAYMOND P. AHLQUIST

TWO DRUGS with similar pharmacological actions when administered simultaneously often act synergistically. This synergism may take the form of a simple algebraic summation of responses or it may appear as a potentiated response. Most quantitative studies of the synergism of centrally acting drugs have been done using the central depressants. The synergism between the convulsant action of morphine and Metrazol, picrotoxin, nikethamide, and strychnine has been reported by Hazleton and Koppanyi (1).

A knowledge of the type of synergism exhibited by the central stimulants is of practical value when they are used therapeutically as analeptics. In treating barbiturate poisoning, for example, a number of different stimulants may be used in the same patient (2). Since many of the stimulants are relatively long-acting it is possible that the presence of one may significantly influence the action of a subsequent stimulant. This study was undertaken to determine the type of synergism occurring between the central stimulants.

GRADATION OF INTENSITY OF STIMULANT ACTION

RESPONSE

A Little observable effect.

B Hyperexcitability and tremors; ears erect; characteristic twitching of nose and ears.

C Metrazol and Strychnine: Posture characteristic; forelegs extended; toes twitching; urination frequent. Coramine: Clonic spasm of hind legs only.

D Severe clonic spasms of 10 to 30 seconds duration; opisthotonus common with Metrazol and Strychnine.

E Severe tonic spasms; death common with Strychnine.

Fig. 1.—Gradation of intensity of stimulant action of metrazol, coramine, and strychnine sulfate when injected intravenously into rabbits.

Metrazol, nikethamide (Coramine), and strychnine sulfate were the stimulants employed.[1] These were chosen because they are commonly used analeptics and because clear, rapid convulsive responses can be obtained on intravenous administration to rabbits. Picrotoxin was not included in this preliminary study because of its delayed convulsive effect which makes it difficult to have its action appear simultaneously with that of another stimulant. No detailed statistical treatment of data was attempted since it was felt that the results obtained were sufficiently clear-cut to render this unnecessary in establishing the general type of synergism present.

METHOD

The stimulant responses to various dosages and combinations of Metrazol, Coramine, and strychnine sulfate were determined in seventy albino rabbits of both sexes and weighing from 1.5 to 2.5 Kg. The drugs were injected rapidly into the marginal ear vein in a volume ranging from 1 to 2 cc. This rapid injection of a small volume was especially important in the case of Coramine since it was found that if larger volumes were injected more slowly the convulsive response was markedly diminished. The stimulant responses were graded as to relative intensity as shown in Fig. 1. The animals were used in groups of ten and no group was used more than four times. Four to ten days were allowed between injections in any one group. In two groups the response to the same dose of Metrazol was tested before and after the series of injections without any significant change in response being noted. All doses are stated on a mg. per Kg. basis.

RESULTS

The dose that would produce type D or E responses in about 50% of the animals tested was first determined for each drug. This dose will be referred

* Received July 31, 1947, from the Department of Pharmacology, University of Georgia School of Medicine, Augusta.
Presented to the Scientific Section, A. PH. A., Milwaukee meeting, August, 1947.
[1] The Metrazol and Coramine were kindly supplied by the Bilhuber-Knoll Corp. and Ciba Pharmaceutical Products, Inc., respectively.

to as the CD_{50}. Figure 2 shows the results obtained using increasing doses of Metrazol. It will be seen that the CD_{50} for Metrazol is about 10 mg. The minimum effective dose (MED) was set as the dose that would produce type C, D, or E responses in one or two animals out of a group of ten. For Metrazol the MED was about 7 mg. The CD_{50} for Coramine was found to be about 50 mg. and the MED about 35 mg. For strychnine sulfate the CD_{50} was found to be about 0.18 mg. and the MED about 0.1 mg. These results agree reasonably well with those reported by Hazleton and Koppanyi (1).

Since innumerable combinations of doses of these three drugs could be used it was necessary to limit this study to a determination of the CD_{50} of combinations when given in equal ratio to their respective CD_{50}'s. Two combinations were tested: Metrazol-Coramine and Metrazol-strychnine sulfate. Starting with a mixture of 5 mg. of Metrazol and 25 mg. of Coramine (50% of each CD_{50}) the doses were increased as shown in Fig. 3. It was found that a mixture of 7 mg. of Metrazol and 35 mg. of Coramine (70% of each CD_{50}) was the CD_{50} for this combination. It was also noted that the convulsions produced by this combination resembled those produced by Metrazol alone rather than those of Coramine.

Treating the combination of Metrazol and strychnine sulfate in the same way it was found that a mixture of 6 mg. of Metrazol and 0.1 mg. of strychnine sulfate (60% of each CD_{50}) produced type D or E responses in about 50% of the animals tested. These convulsions resembled those produced by strychnine sulfate alone.

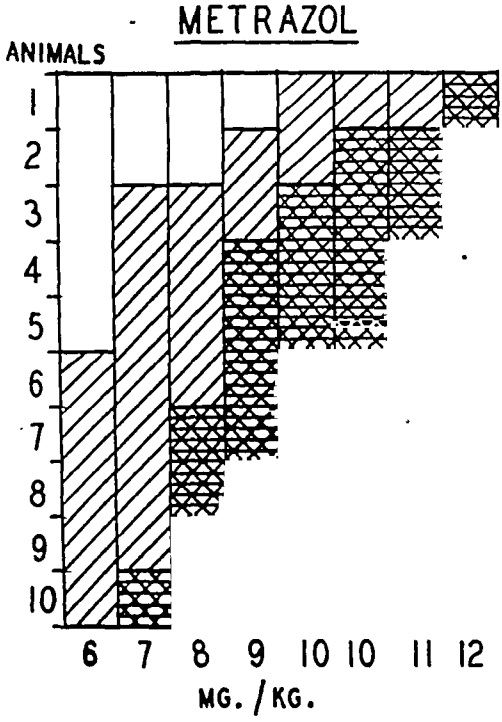

Fig. 2.—Stimulant responses to increasing doses of Metrazol. Gradation of response as shown in Fig. 1.

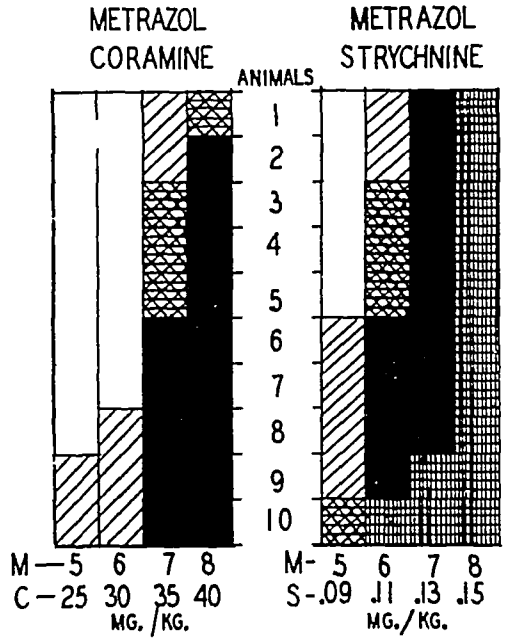

Fig. 3.—Stimulant responses to combinations of Metrazol and Coramine, and Metrazol and strychnine sulfate. Gradation of response as shown in Fig. 1.

DISCUSSION

The type of synergism occurring between these central stimulants is of the deficient additive type when tested on their convulsant actions. The deficiency is probably due to differences in points of action of these substances to produce the convulsions. For example, strychnine acts primarily on the spinal cord whereas Metrazol acts only partially on this structure. Therefore only part of the convulsive effect of the Metrazol would add to the convulsive effect of the strychnine. Another interesting point is that in either combined CD_{50} one of the drugs at least is present in a dosage equal to its MED. This again tends to show that the synergism is of the additive type rather than the potentiated type.

Experimental work should be undertaken to study the synergism of the stimulants in the presence of a depressant. It may well be that a different type of synergism exists between the respiratory stimulating effects or the awakening effects of these analeptics.

SUMMARY

There is an additive synergism between the convulsant effects of the central stimulants Metrazol, Coramine, and strychnine sulfate when tested in rabbits.

REFERENCES

(1) Hazleton, L. W., and Koppanyi, T., *Anesthesiology*, 2, 427(1941).
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A Note on the Hypnotic Principle of *Rauwolfia serpentina**

By J. C. GUPTA, SUDHAMOY GHOSH, A. T. DUTTA, and B. S. KAHALI

In recent years considerable interest has been taken in the drug *Rauwolfia serpentina* Benth ex Kurz belonging to the family Apocynaceae. The plant is fairly widely distributed in India, especially in the sub-Himalayan tracts and in the plains near the foot of the hills. The root of the plant has been used as a home remedy for inducing sleep in children and as a cure for insanity. Instances are known to us in which some types of insanity have been cured by the use of 40 doses of the root powder mixed with powdered black pepper, each dose consisting of one tola (about 11.6 Gm.) of the root and 19 corns of the pepper, administered orally every alternate day.

A number of workers have carried out the chemical investigation of this drug. In India Siddiqui and Siddiqui (1) have isolated five crystalline alkaloids three of which—ajmaline, serpentine, and serpentinine—have been tested pharmacologically. Besides the alkaloids, the roots have been found to contain oleoresins, sterols, unsaturated alcohols, oleic acid, fumaric acid, glucose, sucrose, an oxymethylanthraquinone derivative, a fluorescent substance, and mineral salts (2–10).

The presence of 1.21% to 1.36% of alkaloids in the root naturally diverted the attention of the earlier workers to the alkaloids, which were considered as the only active principles responsible for the hypotensive and hypnotic actions. A standardized alcoholic extract containing about 0.5% of alkaloids was therefore recommended by us for clinical trials, and this extract or tincture manufactured by different commercial firms is used by the medical profession in India for the treatment of hyperpiesis and maniacal types of insanity.

Experiments on frogs showed that the ajmaline group of alkaloids acts as a general depressant to the heart, respiration, and nerves, and the serpentine group of alkaloids paralyzes the respiration and depresses the nerves, but stimulates the heart. Experiments on higher animals showed that both ajmaline and serpentine have a depressant effect on the central nervous system and produce a fall of blood pressure whereas serpentinine causes a rise. None of the alkaloids possessed any marked sedative action whereas the alcoholic extract possessed sedative or hypnotic properties, producing drowsiness, narcosis, and fall of rectal temperature. These observations led us to look for the active principle responsible for the sedative action in the nonalkaloidal components of the drug, and our attention was specially focused on the oleoresin.

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EXPERIMENTAL

The alcohol-soluble extractives of samples from different sources varied widely, e.g., Bengal 4.32, Behar 5.01, Dehra Dun 7.50, and Assam 12.68%. Aqueous extracts of these alcohol-soluble extractives removed all the alkaloids, and the oleoresins left behind were as follows: Bengal 1.14%, Behar 1.25%, Dehra Dun 1.59% and Assam 1.88%. The oleoresins were carefully dried and extracted thoroughly with petroleum ether (b. p. 35–60°) to remove the oily fraction, which when tested pharmacologically proved to have some irritant properties but no hypnotic action. The resin left behind was extracted with 95% alcohol, the soluble portion being found to be active while the insoluble portion was quite inactive. This purified resin, completely freed from alkaloids, water-soluble constituents, and oils, produced sedative and hypnotic effects in experimental animals such as cats, rabbits, guinea pigs, rats, and frogs. The sedative action commences about three to four hours after administration and persists for more than twenty-four hours after administration. It does not cause a fall of blood pressure but stimulates the muscle of the intestine and uterus. This action is produced immediately. The effective dose of the resin for cat and rats was 1 mg. per Kg. of body weight. The present pharmacological and chemical study was confined mainly to the purified total resin, and the action was found to differ quantitatively according to its source. Work on further fractionation of the resin is in progress.

CONCLUSION

The basic importance of the present findings appears to be the fact that no resin other than that of Hemp has been recorded to possess such hypnotic or sedative properties, and an intensive chemical and pharmacological investigation will, we believe, prove to be a very fruitful line of research.

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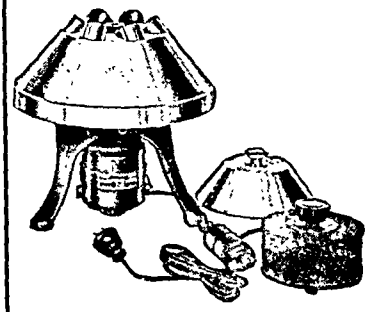

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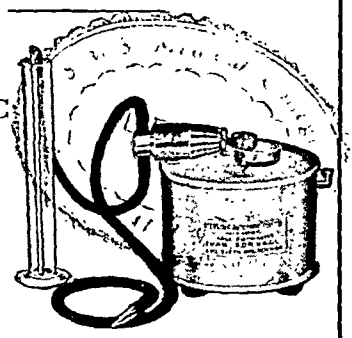

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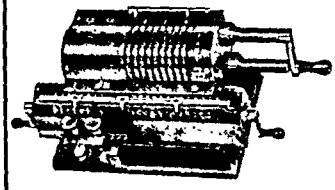

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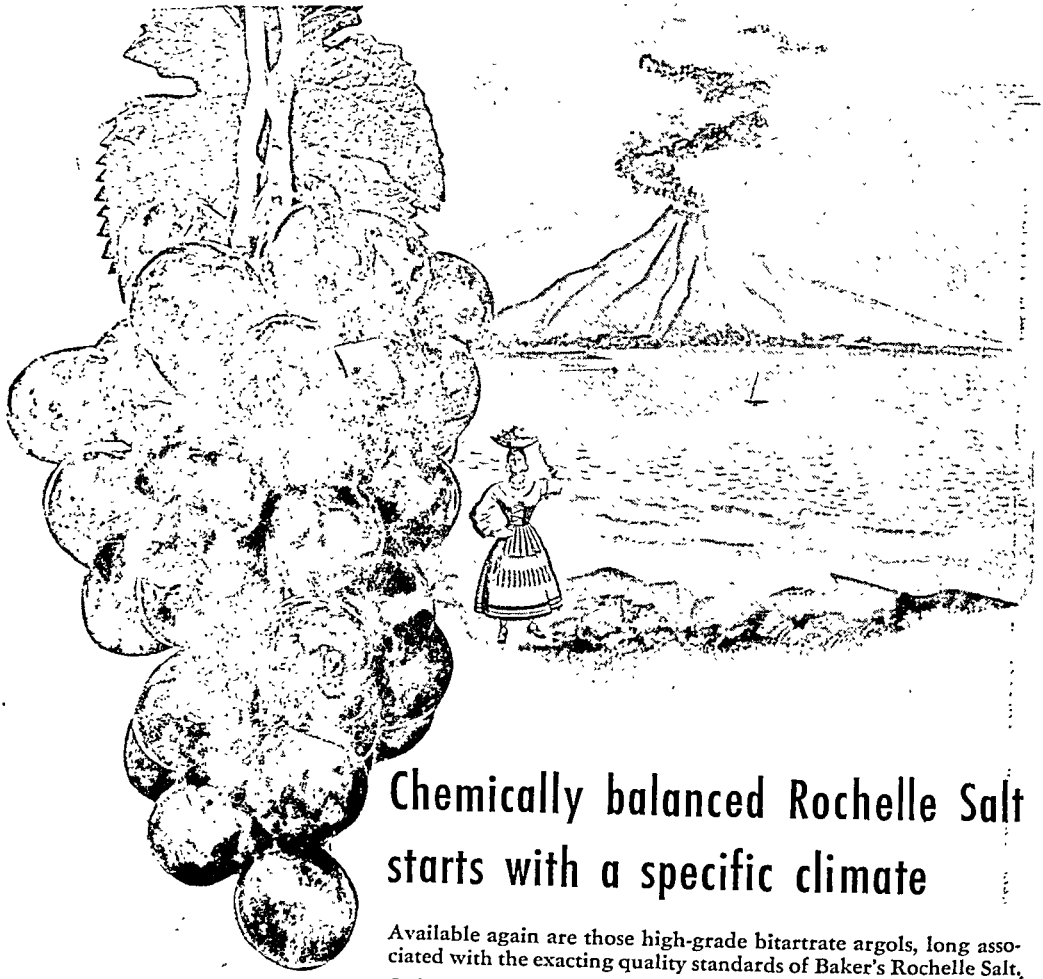

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